10. A method of preparing a frozen product of an iron-containing protein composition solution comprising the step of freezing the iron-containing protein composition solution including a sugar compound of claim 9.

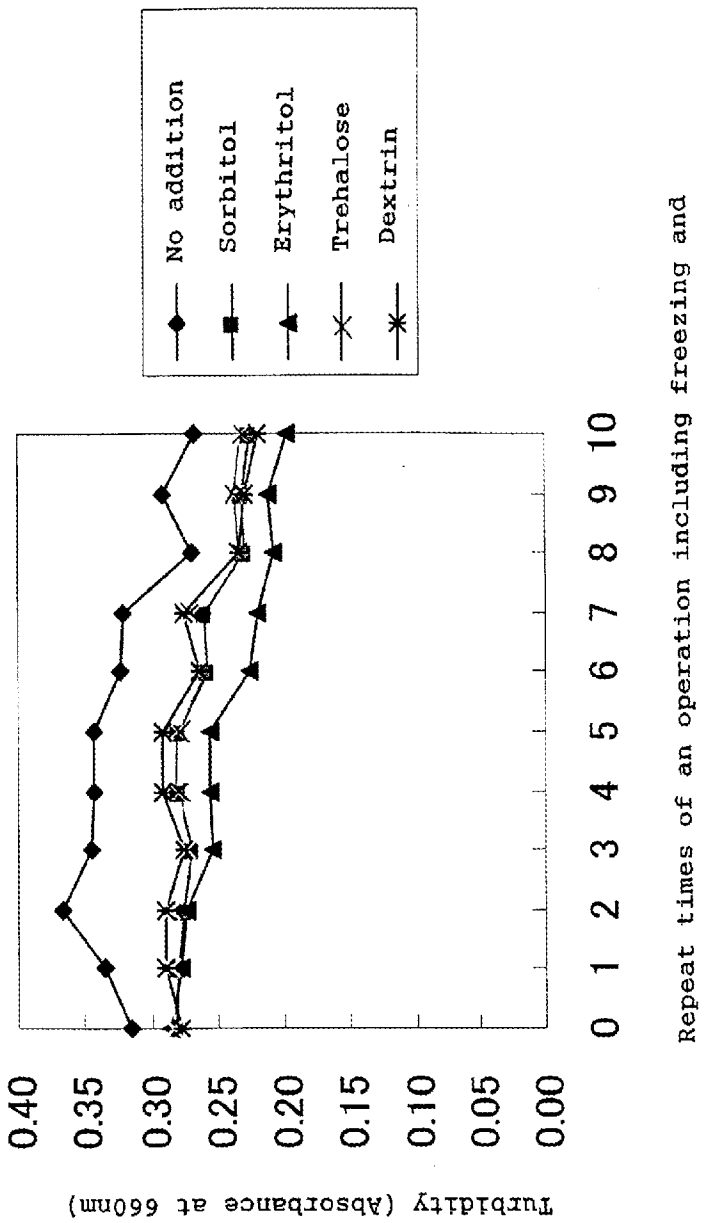
11. A method of preparing a liquid composition comprising the step of thawing a frozen product obtained by the method of Claim 10.

12. A method of preparing a powder comprising the step of drying of the iron-containing protein composition solution of Claim 1.

13. A method of preparing a powder comprising the step of drying of the iron-containing protein composition solution of Claim 2.

14. A method of preparing a powder comprising the step of drying the liquid composition of Claim 4.

Fig. 1


No addition
Sorbitol
Erythritol
Trehalose
Dextrin
Turbidity (Absorbance at 660nm)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 1 2 3 4 5 6 7 8 9 10
Repeat times of an operation including freezing and thawing.

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(54) An improved process for making a cheese product using transglutaminase

(57) The current invention provides an improved process for making a cheese product using transglutaminase. The method comprises the steps of providing a dairy liquid, crosslinking and curding the dairy liquid by adding an acidifying agent and a transglutaminase to the dairy liquid under conditions sufficient to crosslink at least a portion of proteins in the dairy liquid to provide both a curd comprising a crosslinked dairy liquid and a liquid whey, cutting and cooking the curd, separating the curd from the liquid whey, and collecting the curd. Many specific embodiments are provided. Additionally, the current invention provides a product made by the process. The cheese products, especially soft cheeses such cream cheese and cottage cheese, exhibit less syneresis than similar cheeses prepared by conventional methods.

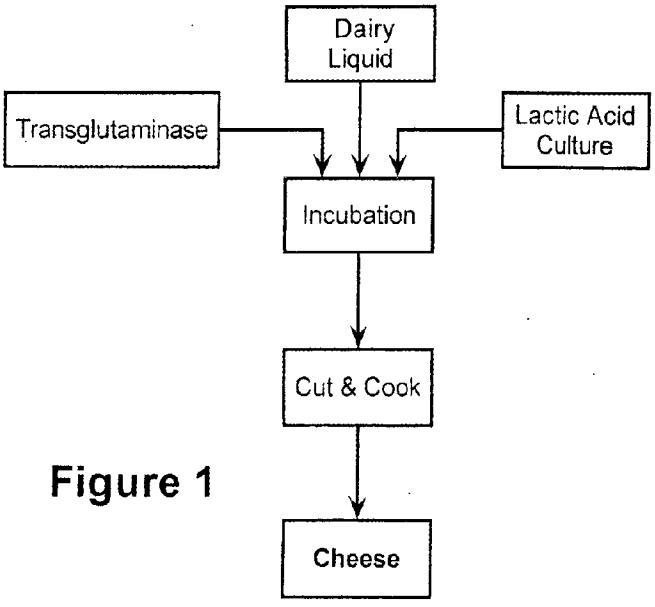


Figure 1

EP 1 254 601 A1

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Description

FIELD OF THE INVENTION

[0001] The invention relates to the production of cheese, especially cream cheese and cottage cheese. More specifically, the invention relates to methods for increasing dairy protein incorporation into curd during cheese production using transglutaminase. This invention also relates to methods for reducing syneresis in cheese products, especially cream cheese and cottage cheese, using transglutaminase.

BACKGROUND

[0002] Cheese compositions are generally prepared from dairy liquids by processes that include treating the liquid with a coagulating or clotting agent. The coagulating agent may be a curding enzyme, an acid, or a suitable bacterial culture. The coagulum or curd that results generally incorporates transformed casein, fats including natural butter fat, and flavorings (especially those arising when bacterial cultures are used). The curd is usually separated from the whey. The resulting liquid whey generally contains soluble proteins not affected by the coagulation; such proteins are, of course, not incorporated into the coagulum. The inability of whey proteins to be retained in the coagulum is an important factor contributing to a lack of efficiency in production of cheese curds, and to a reduction in overall yield relating to the incorporation of protein solids present in the starting dairy liquids into resulting cheese curds. Therefore, there remains a need for effective methods of improving the efficiency of protein incorporation into curd during cheese production.

[0003] Soft cheeses such as cream cheese and cottage cheese products usually suffer water separation during storage due to the process of syneresis. The resulting acid whey (i.e., separated water phase), although it contains substantial amounts of dairy proteins, cannot be efficiently utilized. Moreover, consumers generally find such water separation objectionable. Therefore, there remains a need for improved methods for producing soft cheeses wherein syneresis is reduced.

[0004] Transglutaminases are enzymes which catalyze the crosslinking of proteins. These enzymes have a broad occurrence in nature and may be obtained, for example, from microorganisms such as those belonging to the genus *Streptoverticillium* or from *Bacillus subtilis,* from various Actinomycetes and Myxomycetes, from fish and other marine sources, from plant sources, and from animal sources (especially mammals). Food processing methods employing transglutaminases have been disclosed in recent years. For example, Japanese Patent 59059151 discloses treating an emulsion containing proteins, oils or fats, and water with transglutaminase to produce a gelatinous, crosslinked gel. Japanese Patent 02276541 discloses a heat-resistant food protein having a fibrous texture. The fibrous texture is developed by treatment of a protein hydrogel with a transglutaminase in the presence of calcium ions to induce crosslinking of the surface of a fiber bundle.

[0005] U.S. Patent 5,156,956 discloses a transglutaminase purified from strains of the genus *Streptoverticillium,* and the use of this enzyme to produce gel type foods. This transglutaminase catalyzes formation of protein gelation products from protein solutions to produce conventional gel foodstuffs such as yoghurt, jelly, cheese, gel cosmetics, and the like.

[0006] U.S. Patent 5,731,183 discloses a transglutaminase purified from strains of *Bacillus subtilis*, having particular physical and enzymatic characteristics, and a method for producing protein, peptide, or non-protein amino acid polymers that are crosslinked via their glutamine and lysine residues to form intermolecular or intramolecular conjugates. The crosslinked protein polymers produced using transglutaminase are reported to be useful in a variety of food substances.

[0007] Methods have been proposed for increasing the recovery of whey protein into cheese products using transglutaminase. For example, U.S. Patent 6,093,424 relates to treatment of a dairy liquid such as milk with transglutaminase and a non-rennet protease to generate cheese curd for hard, soft, or semi-soft cheeses.

[0008] U.S. Patent 5,681,598 and its European counterpart EP 0 711 504 A1 disclose a process for producing cheese using transglutaminase added at the same time as both a clotting enzyme and a lactic-acid producing culture, and the milk is then incubated.

[0009] Patent publication WO 97/01961 discloses a method for producing cheese using transglutaminase. Transglutaminase is added to milk, followed by a short incubation period, after which a clotting enzyme or curding agent is added to the milk to produce curd.

[0010] The current invention provides processes for producing cheeses that improve the efficiency of dairy protein incorporation into cheese curd during production. Additionally, the cheeses produced using the process have improved qualities, including decreased syneresis.

SUMMARY OF THE INVENTION

[0011] The current invention provides processes for cheese manufacturing that significantly increase the yield of cheese produced by using the enzyme transglutaminase and an acidifying step to crosslink dairy proteins during cheese manufacturing. The cheese products are less fragile and have reduced syneresis. Additionally, the cheese products have greater viscosity and firm texture.

[0012] In one aspect, the current invention provides a process for making a cheese product comprising:

(i) providing a dairy liquid comprising dairy proteins;
(ii) crosslinking and curding the dairy liquid by adding an acidifying agent and adding a transglutaminase to the dairy liquid for a crosslinking and curding time, and under crosslinking and curding conditions, sufficient to crosslink at least a portion of the dairy proteins and to form a curd and a liquid whey;
(iii) disrupting and heating the curd;
(iv) separating the curd from the liquid whey; and
(v) collecting the curd.

[0013] Typically, a coagulating enzyme is not added during step (ii). In certain embodiments, a coagulating enzyme is not added during any step of the process. The dairy liquid may be pasteurized.

[0014] In certain embodiments, transglutaminase is used in step (ii) at concentrations of less than 10 units per gram dairy liquid, preferably less than 5 units per gram, more preferably less than 1 unit per gram. In certain embodiments, transglutaminase is used at a concentration of about 0.4 to about 0.8 units per gram dairy liquid.

[0015] In one embodiment, the addition of the acidifying agent during step (ii) is performed before the addition of transglutaminase. In a further embodiment, the process further comprises heating the crosslinked dairy liquid at a temperature and for a time sufficient to inactivate the transglutaminase after step (ii) and before step (iii).

[0016] In certain embodiments, the acidifying agent is a culture containing a lactic acid-producing microbe. In one embodiment using a lactic acid-producing microbe, the resulting pH of the dairy liquid is about 4.2 to about 5.2, preferably about 4.4 to about 4.8, and most preferably about 4.5 to about 4.7.

[0017] In certain embodiments, the acidifying agent is a food grade acid. In one embodiment, the food grade acid is selected from the group consisting of citric acid, lactic acid, glucono delta lactone, phosphoric acid, acetic acid or vinegar, and the like.

[0018] In certain embodiments, the dairy liquid comprises dairy ingredients selected from milk, reconstituted dry milk, concentrated milk, milk protein concentrate, whey, reconstituted whey, whey protein concentrate, and cream.

[0019] In certain embodiments, the dairy liquid comprises cream and a dairy ingredient selected from the group consisting of milk, reconstituted dry milk, concentrated milk, milk protein concentrate, whey, reconstituted whey, and whey protein concentrate. For these embodiments, the cheese is typically cream cheese. In one embodiment where the cheese is cream cheese, the cooking in step (iv) is performed by heating the curd and whey to about 80°C for about 30 minutes.

[0020] In certain embodiments, the dairy liquid is skim milk, concentrated skim milk, reconstituted nonfat dry milk, whey protein, or milk protein concentrate. Typically, where the dairy liquid is skim milk, the resulting cheese is cottage cheese. In one embodiment where the dairy liquid is skim milk, the cooking in step (iv) is performed by increasing the temperature of the curd and whey from about 30 to about 60°C over a period of about 15 to about 150 minutes, and more preferably over a period of about 60 minutes. Typically, where the resulting cheese is cottage cheese, the crosslinking and curding time is from about 1 hour to about 14 hours and the crosslinking and curding conditions include a temperature of about 15 to about 55°C, preferably from about 3 hours to about 6 hours at a temperature of about 30 to about 34°C, more preferably at about 32°C.

[0021] Typically, where the resulting cheese is cream cheese, the crosslinking and curding time is from about 1 hour to about 24 hours and the crosslinking and curding conditions include temperatures of about 18 to about 50°C, preferably from about 15 hours to about 19 hours at a temperature of about 22 to about 28°C. In one embodiment, the crosslinking and curding is carried out for about 17 hours at a temperature of about 25°C.

[0022] In significant embodiments of the process of the present invention, the transglutaminase is isolated from a bacterial source, a fungus, a mold, a fish, or a mammal. In one preferred embodiment, the transglutaminase is isolated from a bacterial source, preferably from the genus *Streptoverticillium*. Mixtures of transglutaminase from different sources can be used.

[0023] The processes of the current invention for making cream cheese products may include an additional homogenization step after the curd is collected. The homogenization may be carried out at pressures of up to about 10000 psi (about 68948 kPa), preferably up to about 3500 psi (about 24132 kPa), and most preferably up to about 5000 psi (about 34474 kPa).

[0024] In another aspect, the current invention is a cheese product produced by a process as described above,

including any of the embodiments described above.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] Figure 1 is a flow diagram illustrating the general process of the current invention.

[0026] Figure 2 is a more detailed flow diagram illustrating the preparation of cream cheese using the process of this invention.

[0027] Figure 3 is a more detailed flow diagram illustrating the preparation of cottage cheese using the process of this invention.

[0028] Figure 4 is a Coomassie-stained 16.5% Tricine-SDS PAGE gel of samples obtained during the production of a cottage cheese curd according to Example 3. Lane 1: curd recovered from the transglutaminase-treated milk sample; Lane 2: whey recovered from the transglutaminase-treated milk sample; Lane 3: curd recovered from the control sample; Lane 4: whey recovered from the control sample. Whey and curd samples were loaded with the same quantity of whey and curd, respectively.

[0029] Figure 5 is a graph of the percent concentration of proteins in whey as a function of the final concentration of transglutaminase in milk used in the process according to Example 1.

[0030] Figure 6 contains electron micrographs of cottage cheese curd produced by the method of this invention according to Example 3. Panels A and B illustrate the cottage cheese edge produced with transglutaminase (A) and without transglutaminase (B). Panels C and D illustrate the cottage cheese curd body produced with transglutaminase (C) and without transglutaminase (D).

[0031] Figure 7 is a graph of cream cheese viscosity (Pa) at various pressures of post-curd formation homogenization for samples produced by the process of this invention according to Example 5 using a transglutaminase addition step; a control sample without transglutaminase is also shown.

[0032] Figure 8 is a graph of percent syneresis at various post curd formation homogenization pressures for cream cheese samples produced by the processof this invention according to Example 5 using a transglutaminase addition step; a control sample without transglutaminase is also shown.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0033] The general process of this invention is illustrated in Figure 1. The starting material is a dairy liquid which may or may not be pasteurized. As used herein, "dairy liquid" refers to milk, milk products obtained by fractionating raw milk to provide a liquid fraction, or a solid milk fraction that is reconstituted to a liquid. For example, the milk may be treated to remove some or all of the butterfat, providing low fat milk or skim milk, respectively. Furthermore, whole milk, low fat milk, or skim milk may be concentrated by methods such as evaporation and/or ultrafiltration (with or without diafiltration) and the like. Evaporation provides dairy liquids containing a higher concentration of all the non-volatile components, whereas ultrafiltration provides dairy liquids with a higher concentration of the components that do not permeate the ultrafiltration membrane. In any case, the dairy proteins including casein and whey protein are included among the retained solids, such that their concentrations in the resulting liquids are increased. Furthermore, any of the above dairy liquids may be evaporated to dryness, providing milk solids originating from whole milk, low fat milk, or skim milk, and including casein, whey proteins, and lactose. Any of these solids may be reconstituted by the addition of water or a suitable aqueous composition including milk or a milk fraction. Reconstitution of dry milk thus provides dairy liquids that in general may have a broad range of final concentrations of the component proteins, lactose, butterfat, and other components. All the above liquids are included in the designation of "dairy liquids" as used herein.

[0034] The dairy liquids employed in the present invention may originate from any lactating livestock animal whose milk is useful as a source of human food. Such livestock animals include, by way of nonlimiting example, cows, buffalo, other ruminants, goats, sheep, and the like. Generally, however, cows' milk is the preferred dairy liquid used in the practice of the invention.

[0035] As used herein, "casein" relates to any, or all, of the phosphoproteins in milk, and to mixtures of any of them. An important characteristic of casein is that it forms micelles in naturally occurring milk and in the dairy liquids employed in the present invention. Many casein components have been identified, including, but not limited to, α-casein (including α_{s1} casein and α_{s2} casein), β-casein, K-casein, and their genetic variants.

[0036] As used herein, "whey protein" relates to the proteins contained in the dairy liquid (i.e., whey) obtained as a supernatant of the curds when milk or a dairy liquid containing milk components are curded to produce a cheese-making curd as a semisolid. Whey protein is generally understood to include the globular proteins β-lactoglobulin and α-lactalbumin. It also includes significantly lower concentrations of immunoglobulin, other globulins, and trace amounts of caseins depending on the process of curding and/or whey separation.

[0037] The present invention is especially adapted for, but not limited to, the production of cream cheese and cottage cheese. The dairy liquids used for a given process of the current invention are typically chosen based on the type of

cheese product desired. For example, cream-added dairy liquid is used in the manufacture of cream cheese to balance and/or obtain target compositions in the final product which may be full-fat, fat-free, light, or reduced-fat cream cheese or Neufchatel. A detailed process flowchart illustrating the production of cream cheese using the present invention is shown in Figure 2. As is known in the art, the cream-added dairy liquid for the production of cream cheese products normally has a butterfat content of from less than about 1 up to about 14 percent (and in certain cases up to as much as 20 percent), so that after processing, the finished cream cheese product will have a butterfat content of from less than about 1 up to about 35 percent (and in certain procedures, such as wheyless procedures, up to as much as 38 percent).

[0038] A detailed process flowchart illustrating the production of cottage cheese using the present invention is shown in Figure 3. For the production of such a cottage cheese product, dairy liquids are usually chosen from skim milk, skim milk concentrate, and reconstituted non-fat dry milk.

[0039] For certain embodiments, such as, but not limited to, the production of cream cheese, the dairy liquid is typically homogenized. Homogenization methods are well known in the food sciences. Any homogenization method that can be used to homogenize dairy liquids before processing into a cheese product can be used with the current invention. For example, but not intended to be limiting, cream-added milk for the production of cream cheese can be homogenized in a two-stage homogenizer. Preferably, the first stage is operated at a pressure of about 1000 psi (about 6895 kPa) to about 10000 psi (about 68948 kPa) and the second stage at about 100 psi (about 689 kPa) to about 1000 psi (about 68948 kPa).

[0040] A "pasteurized dairy liquid" is a dairy liquid that has been subjected to pasteurization. The process of pasteurization is well known in the art of food sciences. Pasteurization may or may not be applied in the current invention. Any method for pasteurization can be used for the current invention. For example, but not intended to be limiting, pasteurization of dairy liquids may be carried out by heating the dairy liquid under conventional pasteurization conditions such as, for example, but not limited to, about 72 to about 80°C for at least about 15 seconds.

[0041] Dairy liquids used in the current invention may incorporate other ingredients. For example, calcium may be added for calcium fortification. Suitable calcium sources include, for example, calcium chloride, calcium sulfate, calcium phosphate, calcium citrate, calcium carbonate, calcium gluconate, and the like. Such other ingredients that can be used in the process of manufacturing cheese should not, of course, interfere with transglutaminase activity so as to completely inhibit the ability of this enzyme to crosslink proteins found in dairy liquids.

[0042] In the process of the current invention, the dairy liquids are curded and crosslinked by the addition of an acidifying agent and transglutaminase. For the current invention, crosslinking occurs during the curding process of the cheese-manufacturing process. Typically, the crosslinking agent is added to the dairy liquid at the same time the acidifying agent is added or shortly thereafter. Preferably, the crosslinking agent is added within 1 hour of addition of the acidifying agent, more preferably within 10 minutes of addition of the acidifying agent, and most preferably, immediately after addition of the acidifying agent. Delayed addition of transglutaminase results in a lesser degree of crosslinking with the same enzyme dosage. The degree of crosslinking should be sufficient to provide the desired textural and protein retention characteristics. In the current invention, crosslinking at least a portion of the dairy proteins can be detected using an SDS-PAGE gel and detecting a diminution of main protein bands compared to control, untreated cultures or by detecting a band of aggregates/polymers in SDS-PAGE gels. Preferably, the protein aggregates/polymers remain small enough to enter the stacking gel of the gel. Figure 4 provides a typical gel with curd and whey from the process of this invention as well as curd and whey from a similar process run without transglutaminase.

[0043] Curding and crosslinking are carried out simultaneously and proceed at different rates of reaction during the entire process of curd formation as system conditions change (e.g., changes in pH, lactose content, protein substrates, and the like as the reactions proceed). When lactic acid-producing cultures are used as the acidifying agent, curding and crosslinking are carried out until the pH of the dairy liquid is reduced to a pH of about 4.2 to about 5.2, preferably about 4.4 to about 4.8, most preferably about 4.5 to about 4.7. Typically, the curding and crosslinking are carried out for between 1 and about 24 hours at temperatures of between about 18 and 55°C.

[0044] Typically, where the resulting cheese is cottage cheese, the crosslinking and curding time is from about 1 hour to about 14 hours and the crosslinking and curding conditions include a temperature of about 22 to about 36°C, preferably from about 3 hours to about 6 hours at a temperature of about 30 to about 34°C.

[0045] Typically, where the resulting cheese is cream cheese, the crosslinking and curding time is from about 12 hours to about 24 hours and the crosslinking and curding conditions include a temperature of about 20 to about 30°C, preferably from about 15 hours to about 19 hours at a temperature of about 22 to about 28°C. In one embodiment, the crosslinking and curding is carried out for about 17 hours at a temperature of about 25°C.

[0046] Typically, the curding and crosslinking is done without disturbing the dairy liquid being processed (i.e., without significant agitation). The acidifying agent may be either a food grade acid or a lactic acid-producing culture, both of which are well known in the art. Food grade acids, include, but are not limited to, citric acid, lactic acid, glucono delta lactone, phosphoric acid, acetic acid or vinegar, and the like.

[0047] Preferably, for the current invention, the acidifying agent is a lactic acid-producing culture. Such cultures are

well known in the art of food science. Any lactic acid-producing bacteria used in conventional cheesemaking can be used in the process of the current invention. Not to be limited by theory, as is known in the art, lactic acid-producing microbes are used in cheese manufacturing to ferment lactose present in the dairy liquid and to cause further decomposition of the clotted casein into smaller peptides and free amino acids as a result of the culture's production of proteases and peptidases. The lactic acid-producing culture may be added in amounts which are conventional for the present purpose (i.e., typically about 10,000 to 10,000,000 bacteria/g of dairy liquid). The cultures may be added as freeze-dried, frozen, or liquid cultures.

[0048] As is well known in the art, the lactic acid-producing culture can be added as a starter culture. The starter culture is typically established by inoculating a lactic acid-producing bacteria into a relatively small quantity of dairy liquid compared to the quantity to be used in the cheese production process. The culture is incubated at a temperature that supports multiplication of the bacteria before being used to inoculate the full volume of dairy liquid for the batch of cheese production.

[0049] For the current invention, crosslinking of the dairy liquid occurs while the lactic acid-producing culture is acidifying the dairy liquids. By "crosslinking the dairy liquid " is meant crosslinking proteins present in the dairy liquid. Crosslinking of proteins during the process of the current invention is catalyzed by transglutaminase. Transglutaminases are enzymes which catalyze the transfer of the γ-carboxamide group of a glutaminyl residue in a protein or peptide to the ε-amino group of a lysyl residue of the same or a different protein or peptide, thereby forming a γ-carboxyl-ε-amino crosslink.

[0050] Transglutaminases have a broad occurrence in living systems, and may be obtained, for example, from microorganisms such as those belonging to the genus *Streptoverticillium*, from *Bacillus subtilis*, from various Actinomycetes and Myxomycetes, from fish species and other marine sources, from plant sources, and from animal sources, especially mammals. For example, mammals provide the blood clotting protein activated Factor XIII; liver transglutaminase can be obtained, for example, from pigs. In general, transglutaminases from animal sources require calcium ions for activity. Recombinant forms of transglutaminase enzymes may be obtained by genetic engineering methods as heterologous proteins produced in bacterial, yeast, and insect or mammalian cell culture systems. The principal requirement of any transglutaminase employed in the instant invention is that it has the activity referred to above. Any enzyme having transglutaminase activity may be employed in the methods of the present invention. In a preferred embodiment, the transglutaminase is obtained from the genus *Streptoverticillium.*

[0051] Transglutaminase activity may be determined using known procedures. One such colorimetric procedure uses benzyloxycarbonyl-L-glutaminyl-glycine and hydroxylamine to form a y-carboxyl-hydroxamic acid if transglutaminase is present. An iron complex of the hydroxamic acid can be formed in the presence of ferric chloride and trichloroacetic acid. Using the absorbance at 525 nm with appropriate standards, the activity of enzyme present may be determined (see, e.g.,U.S. Patent 5,681,598 and *J. Biol. Chem.* 240:2951 (1965)).

[0052] In certain embodiments, transglutaminase is used in step (ii) at concentrations of less than 10 units per gram of dairy liquid, preferably less than 5 units per gram, more preferably less than 1 unit per gram of dairy liquid. In certain embodiments, transglutaminase is used at a concentration of about 0.4 to about 0.8 units per gram of dairy liquid.

[0053] After crosslinking and curding, the curd is disturbed and cooked. Typically, the curd is disturbed by cutting or stirring. The processes of cutting or stirring and cooking curd are well known in the art. Examples of cooking conditions are described in the accompanying Examples section. For example, but not intended to be limiting, where the cheese is cream cheese, the cooking can be performed by heating the temperature of the curd and whey to about 80°C for about 30 minutes. In another embodiment where the dairy liquid is skim milk, the cooking in step (iv) can be performed by increasing the temperature of the curd and whey from about 30 to about 60°C over a period of about 60 minutes.

[0054] After being cut and cooked, the curd is separated from the whey and the curd is collected (see, e.g., Option 2 in Figure 2). Methods are well known in the art for separating curd from whey and collecting curd. These methods may vary depending on the type of cheese produced. For example, but not intended to be limiting, centrifugation by a separator (e.g., a Model #KSA6-01-076 separator, Westfalia Separator, Inc., Northvale, NJ.) can be used to separate curd from whey during cream cheese production. Alternatively, the curd and whey can be homogenized without separation (see, e.g., Option 1 in Figure 2).

[0055] Typically, a conventional milk clotting/renneting enzyme is not present during the curding and crosslinking step in the processes of the current invention. In certain embodiments, a conventional milk clotting/renneting enzyme is not present during any step of the process. A "conventional milk clotting/renneting enzyme" is a rennet or a non-rennet protease that has coagulating activity.

[0056] Rennet is a generic term used in the field of dairy science and in the field of cheese making, to designate an activity obtained from the lining of the stomachs of immature mammals that consume maternal milk. The natural function of rennet is to initiate the digestion of the milk in order to provide the nutrition contained in the milk protein to the young mammal. In cheese making, rennet is used to clot the dairy liquids, thereby forming cheese curd and whey. The term "renneting" relates to the process of treating a dairy liquid with a rennet to provide a cheese curd and whey. Synonyms for "renneting" include "curding," "clotting," and "setting." As used in contemporary dairy science, "rennet" connotes

the enzyme earlier called "rennin" and now termed "chymosin." Chymosin is a member of the family of proteases known as aspartyl endopeptidases.

[0057] The activity of chymosin on dairy liquids includes at least the proteolytic cleavage of the peptide bond between the phenylalanyl residue that occurs at about position numbered 105 and the methionine that occurs at about position numbered 106 in κ-casein to release a soluble macropeptide and induce the coagulation of the remainder of the molecule, termed para-κ-casein, with all the components of the casein micelles. Common natural sources of chymosin include, but are not limited to, the stomachs of calves, buffalo, other ruminants, kid goats, lambs, piglets, and the like. Furthermore, various natural chymosins and genetically engineered chymosin mutant proteins are available as the recombinant protein products, obtained as a result of introducing genes encoding these proteins as heterologous genes in order to make the gene products in suitable host organisms. Chymosin is the activated form produced when the proenzyme prochymosin is activated. Prochymosin likewise may be a recombinant product, and may be a genetically engineered mutant protein which upon activation provides renneting activity. As used herein, all such chymosins having renneting activity, and prochymosins activatable to such chymosins, are included in the term "rennet."

[0058] Many other non-rennet enzymes have coagulating activity. Nonlimiting examples include other aspartyl proteases such as various pepsins, and a large number of proteases from nonmammalian sources, including plants, microorganisms, and marine fishes. As used herein, a "non-rennet protease" relates to any such protease having milk-clotting activity that is not a rennet as defined herein. Furthermore, various natural non-rennet proteases, as well as genetically engineered mutant proteins derived from such natural proteases and having the corresponding protease activity, are available as recombinant protein products, obtained upon introducing genes encoding these proteins as heterologous genes into suitable host organisms to produce the protein products. As used herein, all such recombinant non-rennet proteases having milk-clotting activity are included in the term "non-rennet protease."

[0059] Among the non-rennet proteases that may be used in the process of dairy liquid curding are the bacterial protease obtained from *Bacillus licheniformis* and designated SP 446 (Novo Nordisk), the bacterial protease from *Bacillus thermoproteolyticus,* the microbial protease Coralase PN-L produced by *Aspergillus sojae* (Rohm GmbH, Germany), a plant protease such as papain, animal proteases such as a protease from the intestines of fishes.

[0060] The cooked curd produced by the process of the current invention can be combined with other ingredients to obtain final cheese products. These other ingredients include any ingredients typically used in cheese manufacturing. For example, dressings can be added to the curd for the production of creamed cottage cheese (see, e.g., Figure 3).

[0061] The processes of the current invention may include an additional homogenization step after the curd is collected. Preferably, a single- or two-stage homogenizer can be used. Preferably, the first stage is operated at a pressure of about 1000 psi (about 6895 kPa) to about 10000 psi (about 68948 kPa) and the optional second stage at about 100 psi (about 689 kPa) to about 1000 psi (about 6895 kPa).

[0062] In another aspect, the current invention relates to cheese products, especially cream cheese and cottage cheese products, produced by a process as described above, including any of the embodiments described above. The cheese products of the current invention have a number of advantages. For example, the processes of the current invention significantly increase the yield of cheese produced while reducing processing time. The cheese products are less fragile and have reduced syneresis. Additionally, the cheese products have greater viscosity and firm texture. Not to be limited by theory, it is believed that these advantages are the result, at least in part, of smaller, more concentrated protein particles observed in cheese products produced by processes of the current invention (see, e.g., Figure 6).

[0063] The following examples describe and illustrate the processes and products of the invention. These examples are intended to be merely illustrative of the present invention, and not limiting thereof in either scope or spirit. Unless indicated otherwise, all percentages and ratios are by weight. Those skilled in the art will readily understand that variations of the materials, conditions, and processes described in these examples can be used.

EXAMPLE 1: Effect of Transglutaminase Dosage on the Incorporation of Whey Protein into Cottage Cheese.

[0064] An analysis was performed of the effect of transglutaminase on the incorporation of whey protein into cottage cheese. The process used for making cottage cheese consisted of the following steps:

(1) Skim milk was pasteurized at about 72°C for 15 seconds.
(2) A starter culture was prepared by inoculating the pasteurized milk with a lactic acid- producing culture at 22°C and incubating overnight.
(3) The starter culture (6.0 kg) was mixed with 94.0 kg of pasteurized skim milk at 32°C.
(4) During the process of fermentation, transglutaminase was added to the samples at the quantities shown in Table 1.
(5) The samples were incubated at 32°C for about 5 hours to lower pH to about 4.70.
(6) Curd was cut and incubated undisturbed for 30 minutes.
(7) Temperature was increased from 30°C to 60°C over a period of about 60 minutes.

(8) The curd was drained using cheesecloth.

[0065] Protein concentrations were determined using the Lowry assay (Lowry, O.H., Rosebrough, N.G., Farr, A.L., and Randall, R.J., "Protein Measurement with Folin phenol reagent," *J. Biol. Chem.* 193:265 (1951)). Transglutaminase activity was determined as described in *J. Biol. Chem.* 240:2951 (1965). One unit of transglutaminase activity is defined as the amount of enzyme that catalyzes the formation of 1 micromole of hydroxymate per minute under the assay conditions.

[0066] Experimental results are summarized in Table 1. The concentration of proteins in the recovered whey decreased with increasing dosage of transglutaminase. Compared to the control sample which had no added transglutaminase, up to 26 percent of whey protein was incorporated into cottage cheese curd. Figure 5 shows the relationship between the activity of transglutaminase added to milk and the concentration of proteins in the whey. Of course, decreasing whey protein in the whey corresponds to increasing whey protein in the curd. In addition to the incorporation of whey proteins into curd that caused a significant increase in the yield of curd, the firmness of cottage cheese curd was also significantly increased by the reaction of transglutaminase cross-linkage.

Table 1.

Effect of Transglutaminase (TG) Dosage on the Incorporation of Whey Protein into Cottage Cheese			
No.	Sample	Proteins in Whey (A_{660} nm)*	Proteins in Whey (%)
	Milk (g)	TG (g)	
1	225	0	0.30740.484
2	225	0.40	0.2564 0.404
3	225	0.80	0.2339 0.368
4	225	1.40	0.2257 0.355

*Whey recovered was diluted 10X for the assay. Data are mean values of triplicate measurements.

Example 2. Denatured Transglutaminase Has No Affect on Whey Protein Incorporation.

[0067] An analysis was performed to determine whether the effects of transglutaminase on the incorporation of whey proteins into cottage cheese required the transglutaminase enzyme to be active. The process for making cottage cheese was carried out as described in Example 1 except that either denatured or active transglutaminase was added to the culture as shown in Table 2. Additionally, the formed curd was cut after about 4 hours incubation at 32°C instead of about 5 hours incubation as in Example 1. Protein concentration and transglutaminase activity was measured as described in Example 1.

[0068] Results of the experiment are summarized in Table 2. This experiment further confirms that the decreased protein concentration in recovered whey is caused by the cross-linking of transglutaminase. Heat denatured transglutaminase had no effect on the incorporation of whey protein into cheese curd. The concentration of proteins in whey recovered from the sample with the addition of heat denatured transglutaminase was the same as that of control samples, indicating that the components in the inactivated enzyme sample had no effect on whey protein incorporation. On the other hand, up to 23 percent of whey protein was incorporated into cottage cheese curd with the addition of 0.60 g of active transglutaminase (100 units/g).

Table 2.

Experimental Design and Results			
No.	Sample	Proteins in Whey (A_{660} nm)*	Proteins in Whey (%)
	Milk (g)	TG (g)	
1	400	0	0.73670.464

*Whey recovered was diluted 5X for the assay. Data are mean values of triplicate measurements.

Table 2. (continued)

Experimental Design and Results			
No.	Sample	Proteins in Whey (A_{660} nm)*	Proteins in Whey (%)
	Milk (g)	TG (g)	
2	400	0.60 (denatured)	0.7361 0.464
3	400	0.60	0.5577 0.351
4	400	0.60	0.5678 0.358
5	400	0.60	0.5776 0.364

*Whey recovered was diluted 5X for the assay. Data are mean values of triplicate measurements.

Example 3. Pilot Plant Testing of the Process for Incorporating Whey Protein into Cottage Cheese.

[0069] An analysis was performed of the ability of the process of the current invention to increase the amount of protein incorporated in cottage cheese when carried out in a larger batch size. The process used was a modified version of the process described in Example 1. The pilot plant process used for making cottage cheese consisted of the following steps:

(1) Skim milk was pasteurized at about 72°C for 15 seconds.
(2) A starter culture was prepared by inoculating the pasteurized milk with a lactic acid- producing culture at 22°C and incubating overnight.
(3) The starter culture (12.7 kg) was mixed with 215 kg of pasteurized skim milk at 32°C.
(4) During the process of fermentation, transglutaminase (0.68 kg enzyme powder dissolved in 2.8 kg water) was added to the sample and the sample was mixed.
(5) The samples were incubated at 32°C for about 4 hours to lower pH to about 4.70.
(6) Curd was cut and incubated undisturbed for 30 minutes.
(7) Temperature was increased from 30°C to 55°C over a period of about 120 minutes.
(8) The curd was drained then washed with water twice.
(9) The washed curd was drained, weighed, and mixed with dressing (prepared as shown diagrammatically in Figure 3) to make the final cottage cheese product.

[0070] Control samples were processed as above except that no transglutaminase was added. The activity of the transglutaminase used in this experiment was 100 Unit/g enzyme powder. Moisture content was determined using a vacuum oven test. For this test, the weight of curd was determined before and after drying the curd in a vacuum oven.
[0071] Protein concentrations and transglutaminase activity were determined as described in Example 1. Whey samples were filtered to remove fine particles and diluted 10X before determining their protein concentration by the Lowry assay. Protein composition of curd and whey fractions were analyzed by Coomassie blue R-250 staining of proteins separated by electrophoresis on 16.5% SDS polyacrylamide gels, according to the method of Laemmli, U.K., *Nature,* 227:680 (1970). A typical gel is shown in Figure 4 for both inventive curd and whey and for the control curd and whey.
[0072] Transmission electron microscopic analysis was performed using standard procedures known in the art, as described in HAYAT's *Principles and Techniques of Electron Microscopy,* Litton Educational Publishing, Inc. The samples were counterstained with osmium tetroxide (OSO_4) and analyzed using a ZEISS model EM10 transmission electron microscope. Figure 6 provides typical electron micrographs of cottage cheese curd produced.
[0073] Results of the experiment are summarized in Table 3. Compared to control samples (i.e., no added transglutaminase), 23 percent of whey protein was incorporated into the cottage cheese curd in samples containing added transglutaminase. The incorporation of whey proteins into curd caused a significant increase in the yield of curd (39.6 kg vs. 31.7 kg). Total curd solids recovered from transglutaminase-treated milk was 26 percent greater than that from control sample. The application of transglutaminase to cottage cheese production, therefore, yields significant productivity savings.

Table 3.

Differences Between Transglutaminase-treated Sample and Control		
	Transglutaminase Treated	Control
Proteins in Whey (A_{660} nm)*	0.212	0.276
Curd Obtained (kg)	39.6	31.7
Moisture in Curd (%)	78.1	78.4
Total Solids in Curd (kg)	8.67	6.85

* Protein content in the whey samples was 3.87% for the control sample and 2.97% for the transglutaminase-treated sample, according to this assay. Data are mean values of triplicate measurements.

[0074] In addition to the increase in curd yield, the texture of cottage cheese curd was significantly improved (i.e., increase in firmness) by transglutaminase cross-linking. Analysis of the microstructure of curd samples indicated that transglutaminase-treated curd contained smaller protein particles with less empty space between the particles (see Figure 6). This reduced empty space (both at the edges and the body of the curd) likely contributes to the reduced rate of syneresis and the unique texture of the cottage cheese of this invention. Thus, it appears that transglutaminase cross-linking significantly effects both the microstructure of the curd and the incorporation of whey protein in the curd. It also appears that the less-fragile curd resulted in fewer fine particles of curd being lost in the whey. A cooked egg-white-like texture was produced with the cross-linking using transglutaminase. The texture is very unique for curd. The significantly firmer texture of curd formed from transglutaminase-cross-linking reduces the need for gentle handling of curd during the initial stages of cooking, resulting in a more robust process.

[0075] Figure 4 shows the protein profile of curd and whey recovered from the pilot plant process and a control process without added transglutaminase. Compared to whey recovered from control samples, whey recovered from transglutaminase-treated milk contained fewer proteins, especially caseins. Based on the small amount of proteins remaining in the whey of transglutaminase-treated samples (Figure 4, lane 2), it may be difficult to incorporate further β-lactoglobulin and/or α-lactobumin into curd. Transglutaminase treatment produced protein polymers with a wide range of molecular weights (Figure 4, lane 1). These polymers likely contribute to the unique texture of the cottage cheese curd obtained. The texture of cheese curd, therefore, can be adjusted by controlling cross-linking conditions.

EXAMPLE 4: Effect of Transglutaminase Dosage on the Incorporation of Whey Protein into Cream Cheese.

[0076] An analysis was performed of the effect of transglutaminase on the incorporation of whey protein into cream cheese. The process used for making cream cheese consisted of the following steps:

(1) Cream-added milk was homogenized in two stages at a pressure of 1600 psi (11032 kPa) in the first stage and 500 psi (3447 kPa) in the second stage and pasteurized at about 72°C for 15 seconds. The milk/cream ratio was 0.743 / 0.257. The full fat milk contained 12.0% solids, 88.0% moisture, 3.6% fat, and 3.2% protein. The cream contained 45.5% solids, 54.5% moisture, 41.0% fat, and 2.0% protein.
(2) The pasteurized cream-added milk was inoculated with 0.75 percent volume of a bulk culture of a lactic acid-producing culture at 25°C.
(3) During the process of fermentation, transglutaminase was added to the samples at the quantities shown in Table 4.
(4) The samples were incubated without disturbance at about 25°C for about 17 hours to lower pH to about 4.50.
(5) The coagulum was stirred and the sample was heated to 80°C and held for 30 minutes.
(6) The curd was separated from the whey by centrifugation at 1500 rpm for 10 minutes.

[0077] Protein concentrations and transglutaminase activity was determined as described in Example 1. Recovered whey was diluted 10X for protein concentration determinations. Control samples were prepared using a process identical to that described above except that no transglutaminase was added or the added transglutaminase (20 percent w/v) was heat denatured by heating to 85°C for 10 minutes before addition to the pasteurized milk. The percent of whey protein incorporated into transglutaminase-added samples was determined by subtracting the A_{660} of whey samples from processes where active transglutaminase was used from the A_{660} of whey samples from processes where heat-treated transglutaminase was used and dividing the remainder by 0.373.

[0078] Results of the experiment are summarized in Table 4. The concentration of proteins in the recovered whey decreased with increasing dosage of transglutaminase. Compared to control samples without added transglutaminase,

up to 19 percent of whey protein was incorporated into cream cheese curd. Heat denatured transglutaminase had no effect on the incorporation of whey protein into cheese curd. The concentration of proteins in whey recovered from the sample with the addition of heat denatured transglutaminase was the same as that of control samples without added transglutaminase, indicating that the components in the inactivated enzyme sample had no effect on whey protein incorporation. A number of benefits were found with the addition of transglutaminase during the cream cheese manufacturing process, in addition to the increased utilization of starting milk protein. For example, the incorporation of whey proteins into curd caused an increase in the yield of curd. In addition, the firmness and viscosity of cream cheese curd was significantly increased by the transglutaminase cross-linkage.

Table 4.

Effect of Transglutaminase (TG) Dosage on the Incorporation of Whey Protein into Cream Cheese

No.	Sample		Proteins in Whey	Whey Protein Incorporated
	Milk (g)	TG (g)	(A_{660} nm)*	(%)
1	500	0	0.373	0.0
2	500	0.40 (heated)	0.375	0.0
3	500	0.20	0.347	7.0
4	500	0.40	0.322	14.2
5	500	0.80	0.315	18.2
6	500	1.50	0.331	19.3
7	500	1.50 (heated)	0.403	0.0

* Whey recovered was diluted 10 X for the assay. Data are mean values of triplicate measurements.

Example 5. Pilot Plant Testing of the Process for Incorporating Whey Protein into Cream Cheese.

[0079] An analysis was performed of the ability of the process of the current invention to increase the amount of protein incorporated in cream cheese, when carried out in a larger batch size. The process used was a modified version of the process described in Example 4. The pilot plant process used for making cream cheese consisted of the following steps:

(1) Cream-added milk (1362 kg total, 997 kg milk mixed with 365 kg cream) was homogenized in two stages, at a pressure of 1600 psi (11032 kPa) in the first stage and 500 psi (3447 kPa) in the second stage, and pasteurized at or above 72°C for 15 seconds.
(2) The pasteurized cream-added milk was inoculated with 0.75 percent volume of a bulk culture of a lactic acid-producing culture at 25°C.
(3) During the process of fermentation, 2.0 kg of transglutaminase dissolved in 10 kg of water was added to the sample.
(4) The samples were incubated without disturbance at about 25°C for about 17 hours to lower pH to about 4.50.
(5) The mixture was heated to about 82°C by pumping it to a plate heat exchanger.
(6) The mixture was then pumped to a separator (Model #KSA6-01-076, Westfalia Separator, Inc., Northvale, NJ) and separated at 82°C.
(7) A slurry of whey, salt, and/or gum mixture was added to the separated curd and moisture was adjusted to 60 percent.
(8) The sample was homogenized at 0 psi (0 kPa), 1500 psi (10342 kPa), 3500 psi (24132 kPa), and 5000 psi (34474 kPa).
(9) The sample was hot filled and stored at 4°C after 15 minutes incubation after filling.

[0080] Protein concentrations in the cheese samples were determined using the Kjeldahl method (Bradstreet, R.B., "The Kjeldahl Method for Organic Nitrogen," Academic Press, Inc., New York, NY (1965); Official Methods of Analysis, 14th Ed., AOAC, Arlington, VA, Methods No. 2.057, 24.038 - 24.040 (1985)) using a KJELTEC AUTO 1030 Analyzer (Fisher Scientific, Itasca, IL).

[0081] Protein concentrations in the whey samples recovered were determined using the Lowry assay (Lowry, O.H.,

Rosebrough, N.G., Farr, A.L. and Randall, R.J., "Protein measurement with Folin phenol reagent," J. *Biol. Chem.* 193: 265 (1951)). Transglutaminase activity was determined as described in Example 1.

[0082] Recovered whey was diluted 10X for protein concentration determinations. Control samples were prepared using a process identical to that described above except that no transglutaminase was added. The percent of whey protein incorporated into transglutaminase-added samples was determined by subtracting the A_{660} of whey samples from processes where active transglutaminase was used from the A_{660} of whey samples from processes where heat-treated transglutaminase was used and dividing the remainder by 0.274.

[0083] Moisture content was determined using a vacuum oven test. For this test, the weight of curd was determined before and after drying the curd in a vacuum oven.

[0084] Viscosity of the samples was measure at 4°C using a Viscometer (Model HAAKE VT550, HAAKE Inc., Karlsruhe, Germany). Syneresis was measured after a 20-hour incubation at room temperature by determining the amount of moisture phase separated from the sample during the incubation period. For this measurement, the cheese sample in an 8 oz (236.6 ml) cup was cut in the middle and half of the sample removed completely to the bottom of the cup and its weight measured. After incubation, the moisture phase was removed and the net change in weight is the amount of moisture separated. The rate of syneresis = net change in weight divided by the original weight of the sample after cutting.

[0085] Fat concentration was determined by a standard method as described in the AOAC 16th Ed., 4th Revision, March 1998, method #33.7.17. Lactic acid concentration was determined by using an enzyme/spectrophotometric assay with a Boehringer Mannheim (Indianapolis, IN) test kit.

[0086] The process used for making cream cheese in the pilot plant for this experiment is shown in Figure 9. Results of analysis of whey remaining after the manufacture of cream cheese using this pilot plant process are shown in Tables 5 and 6. Compared to control samples, which were manufactured without the addition of transglutaminase, 13.5 percent of whey protein was incorporated into cream cheese curd for the sample treated with transglutaminase (Table 5).

[0087] In addition to the incorporation of whey proteins into curd which caused an increase in the yield of curd, the texture/viscosity of the cream cheese curd was also significantly improved by the transglutaminase cross-linkage. Without post homogenization, the viscosity of transglutaminase-treated samples was 5.5 times greater than that of control samples. Cross-linking of proteins also minimized syneresis of the final product. The syneresis rate of the final product was decreased about 50 percent in the cream cheese curd prepared with the addition of transglutaminase. The increase in product viscosity and decrease in syneresis were further confirmed by repeated pilot plant experiments (data not presented). With the incorporation of whey protein as well as increased moisture in the sample of transglutaminase-treated milk, its fat content was lowered. In addition, the transglutaminase-treated sample contained less lactic acid, which should improve the flavor of the final product.

Table 5.

Incorporation of Whey Protein into Curd by Transglutaminase (TG) Treatment		
Sample	Proteins in Whey (Average*; A_{660} nm)	Whey Protein Incorporated (%)**
Transglutaminase Treated	0.237	13.5
Control Sample	0.274	0.0

*Data are mean values of duplicated measurements.

** The rate of whey protein incorporation = (A_{660} control - A_{660} TG-treated)/0.274.

Table 6.

Characteristics of Soft Cream Cheese Prototypes from Pilot Plant Experiment*								
	Homog. (Psi)[kPa]	Moisture (%)	Viscosity (Pa)	Syneresis (%)	Fat (%)	Acidity (%)	Lac. Acid (%)	Proteins (%)
A	0[0]	61.1	3637	6.0	28.2	0.74	0.46	6.3
	5000[34474]	60.4	5912	5.5	28.8	0.71	0.42	6.3
B	0[0]	58.9	656	11.1	31.0	0.79	0.49	5.9
	5000[344741	58.4	5226	10.2	31.9	0.78	0.51	6.3

* Data are mean values of duplicate measurements.

[0088] In addition to the effects of transglutaminase addition, the process of homogenization of the curd had significant effects on product texture and syneresis. As shown in Figure 7, product viscosity increased with increasing homogenization pressure as expected. In addition, as mentioned above the viscosity of transglutaminase-added products was greater than that of control samples. In addition, the viscosity of transglutaminase-treated samples was almost linearly proportional to the pressure of homogenization, indicating that cross-linked protein conjugates are compatible with the fat globule and curd matrix.

[0089] The homogenization pressure applied to formed curd, however, had fewer effects on the rate of product syneresis. No significant change on the rate of syneresis was observed for control samples or transglutaminase-treated samples with increasing homogenization pressure (Figure 8). As found in experiments described in previous Examples, transglutaminase-treated samples had reduced syneresis compared to control samples. The results reported in this Example support the conclusion that the process of the current invention produces firmer cream cheese products with minimized syneresis.

[0090] Throughout this application, various patents, publications, books, nucleic acid and amino acid sequences have been cited. The entireties of each of these patents, publications, books, and amino acid sequences are hereby incorporated by reference into this application.

Claims

1. A process for making a cheese product comprising:

   (i) providing a dairy liquid comprising dairy proteins;
   (ii) crosslinking and curding the dairy liquid by adding an acidifying agent and a transglutaminase to the dairy liquid for a crosslinking and curding time, and under crosslinking and curding conditions, sufficient to crosslink at least a portion of the dairy proteins and to form a curd and a liquid whey;
   (iii) disturbing and heating the curd;
   (iv) separating the curd from the liquid whey; and
   (v) collecting the curd.

2. A process according to claim 1, wherein the acidifying agent is added before or after the transglutaminase during step (ii).

3. A process according to claim 1 or claim 2, wherein the process further comprises heating the crosslinked dairy liquid after step (ii) and before step (iii) at an inactivation temperature and an inactivation time sufficient to inactivate the transglutaminase.

4. A process according to any one of claims 1 to 3, wherein the acidifying agent is a culture containing a lactic acid-producing microbe and no conventional/milk clotting/renneting enzyme is used in the process.

5. A process according to any one of claims 1 to 3, wherein the acidifying agent is a food grade acid and no conventional/milk clotting/renneting enzyme is used in the process.

6. A process according to any one of claims 1 to 5, wherein the crosslinking and curding time is the time required to bring the pH of the dairy liquid to a pH of about 4.2 to about 5.2 .

7. A process according to claim 6, wherein the cheese is a cream cheese and the dairy liquid comprises cream and a dairy ingredient selected from milk, reconstituted dry milk, concentrated milk, whey, whey protein concentrate, and milk protein concentrate.

8. A process according to claim 6 or claim 7, wherein the cheese is a cream cheese and the crosslinking and curding time is from about 1 hour to about 24 hours and the crosslinking and curding conditions comprise a temperature of about 18 to about 55°C.

9. A process according to claim 7 or claim 8, wherein in step (iii) the heating is performed by bringing the temperature of the curd and whey to about 55 to 80°C for about 1 to 60 minutes.

10. A process according to claim 6, wherein the cheese is a cottage cheese and the dairy liquid is selected from the group consisting of milk, concentrated skim milk, reconstituted nonfat dry milk, whey protein, and milk protein

concentrate.

11. A process according to claim 6 or claim 10, wherein the cheese is a cottage cheese and the crosslinking and curding time is from about 1 hour to about 14 hours and the crosslinking and curding conditions comprise a temperature of about 15 to about 55°C.

12. A process according to claim 10 or claim 11, wherein in step (iii) the heating is performed by increasing the temperature of the curd and whey from about 30 to about 60°C over a period of about 15 to about 250 minutes.

13. A process according to any one claims 1 to 12, wherein the transglutaminase is selected from a group of transglutaminases isolated from a bacterial source, a fungus, a mold, a fish, and a mammal.

14. A cheese product provided by a process according to any one of claims 1 to 13.

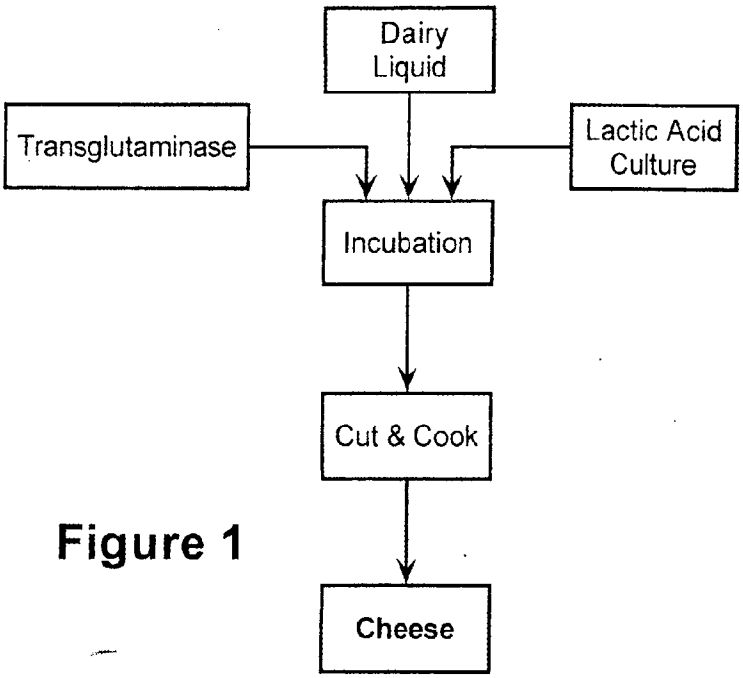

Dairy Liquid
Transglutaminase
Lactic Acid Culture
Incubation
Cut & Cook
Cheese

Figure 1

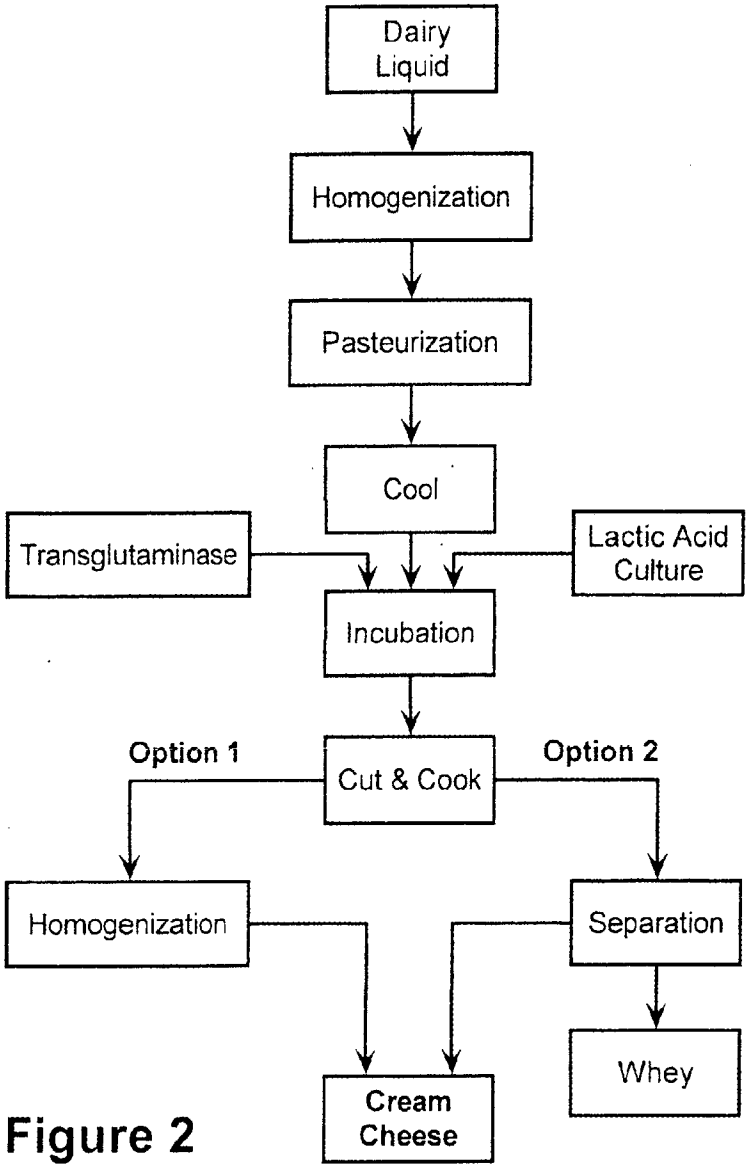

Dairy Liquid
Homogenization
Pasteurization
Cool
Transglutaminase
Lactic Acid Culture
Incubation
Option 1
Cut & Cook
Option 2
Homogenization
Separation
Whey
Cream Cheese

Figure 2

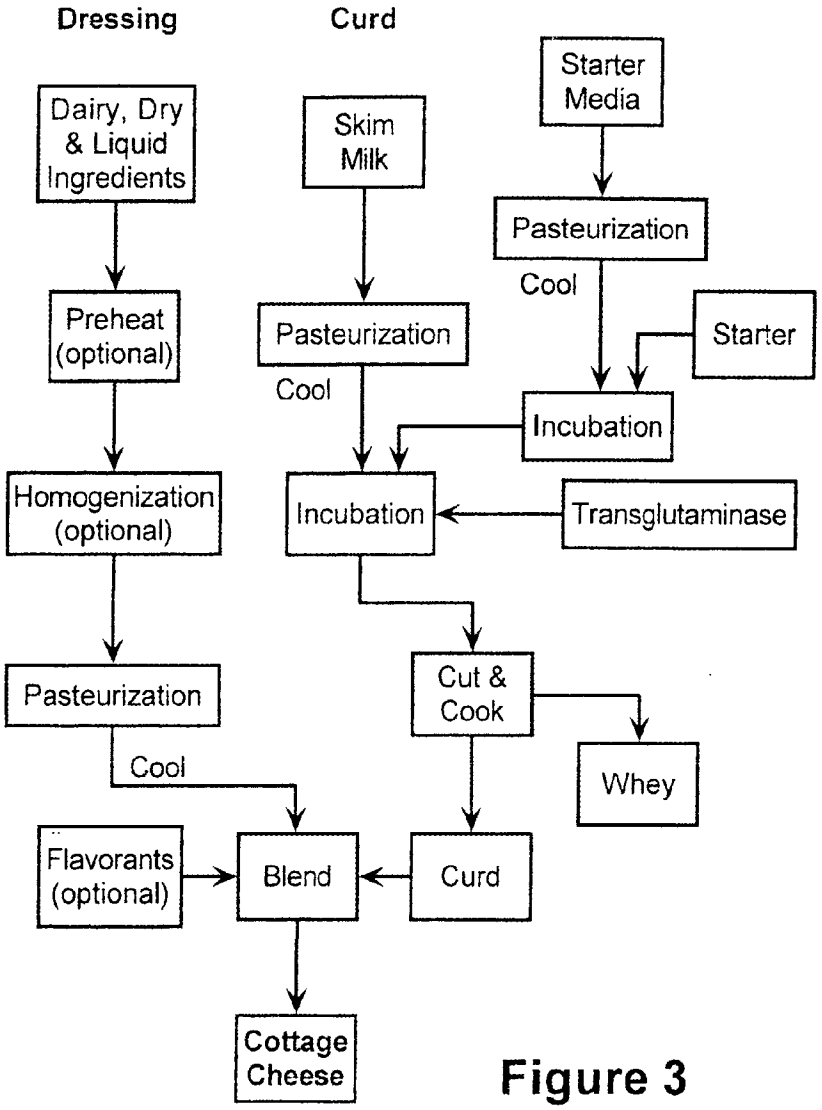

Dressing
Curd
Dairy, Dry & Liquid Ingredients
Skim Milk
Starter Media
Pasteurization
Cool
Preheat (optional)
Pasteurization
Cool
Starter
Incubation
Homogenization (optional)
Incubation
Transglutaminase
Pasteurization
Cut & Cook
Cool
Whey
Flavorants (optional)
Blend
Curd
Cottage Cheese

Figure 3

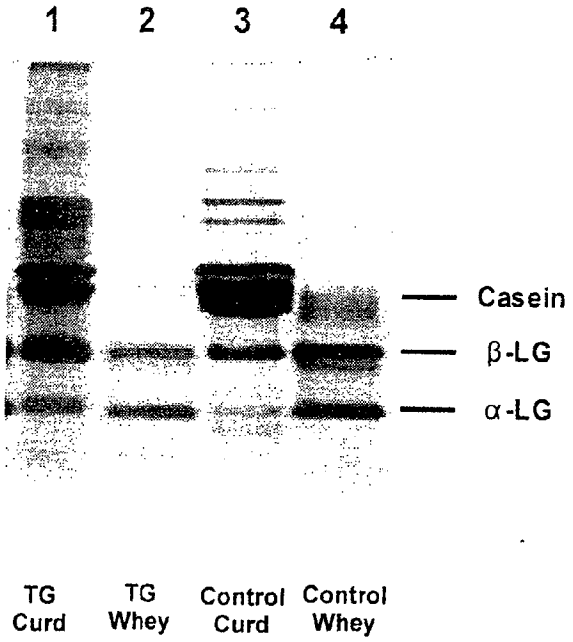
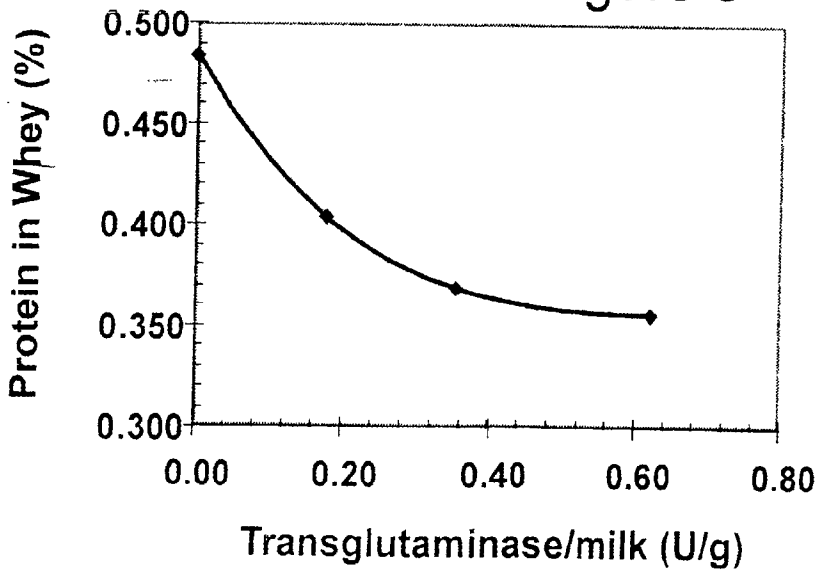


Figure 4

Figure 5



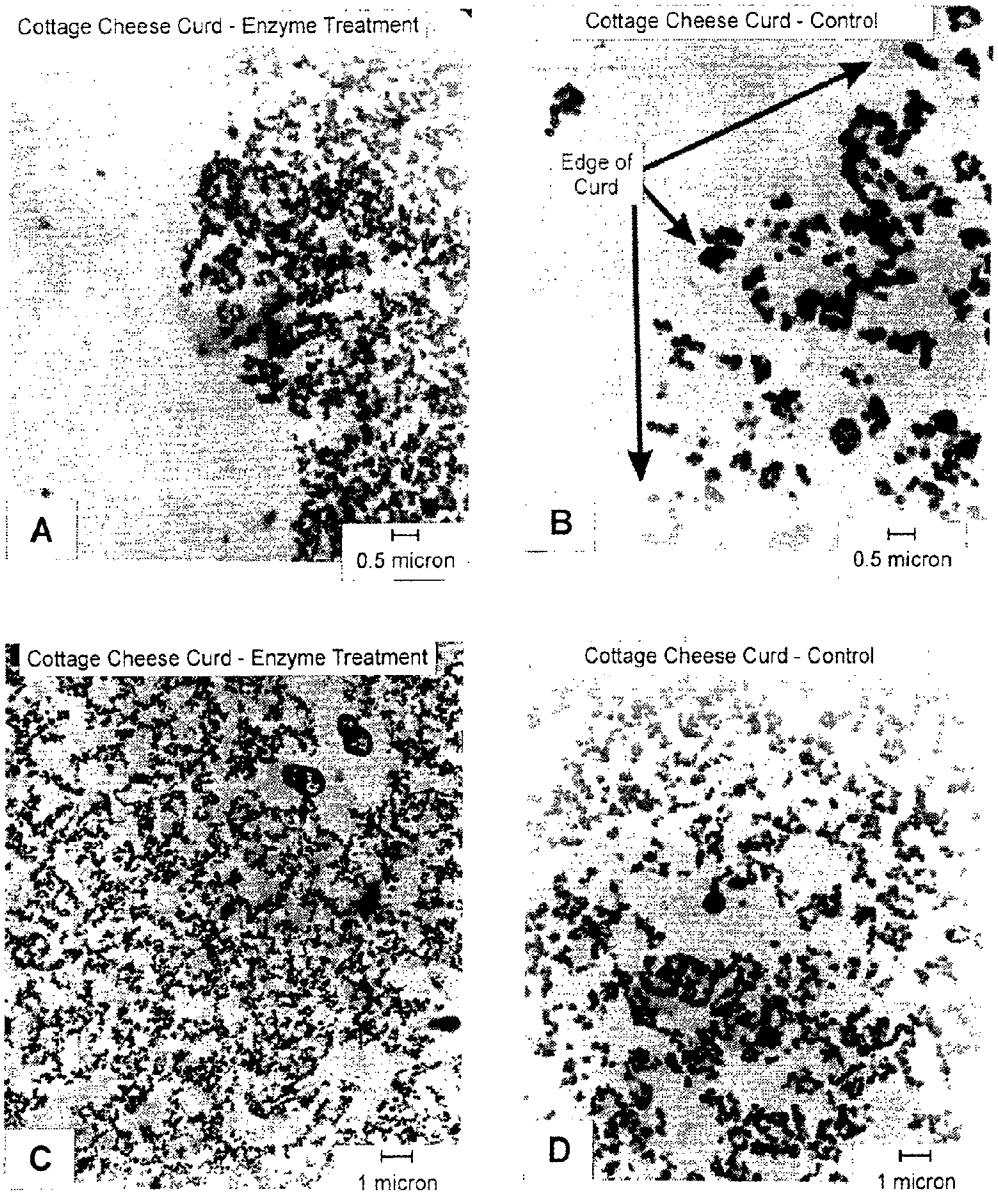

Cottage Cheese Curd - Enzyme Treatment
A
0.5 micron
Cottage Cheese Curd - Control
Edge of Curd
B
0.5 micron
Cottage Cheese Curd - Enzyme Treatment
C
1 micron
Cottage Cheese Curd - Control
D
1 micron

Figure 6

Figure 7

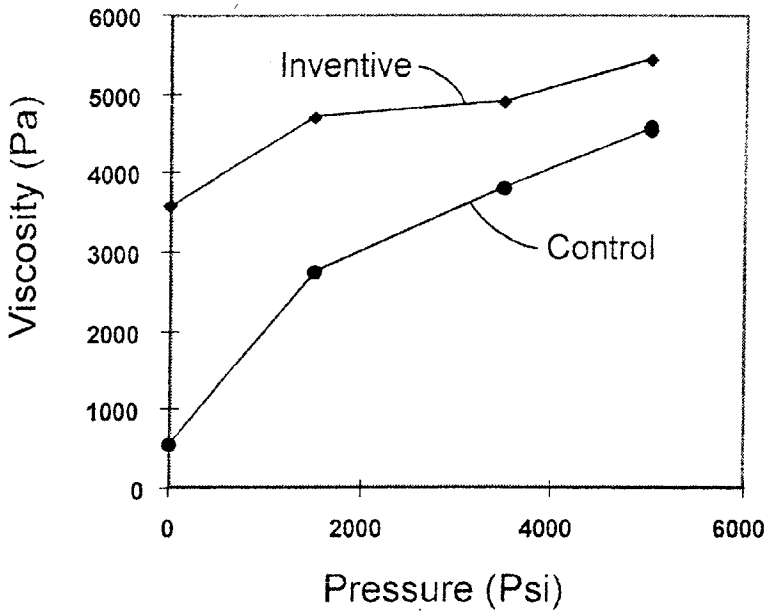
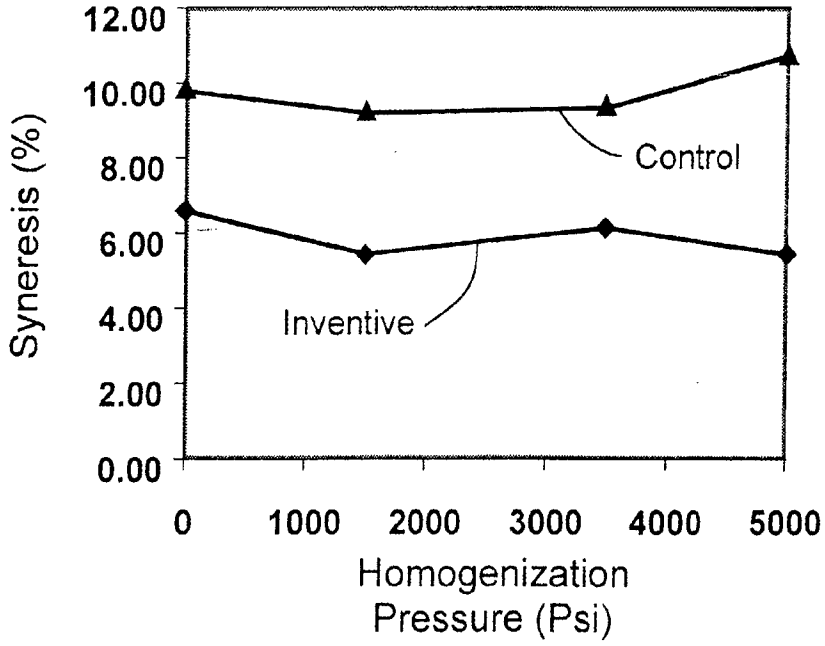


Figure 8



European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 02 25 3037

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	19 July 2002	Koch, J

CATEGORY OF CITED DOCUMENTS

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(54) PRODUCTS CONTAINING G(b)-GLUCAN

PRODUKTE ENTHALTEND G(b)-Glucan

PRODUITS CONTENANT DU G(B)-GLUCANE

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Description

Technical Field:

[0001] The present invention relates to a fat and oil composition containing β-glucans extracted from a plant of the Gramineae family. More particularly, it relates to an edible fat and oil composition which is uniformly dispersible in fats and oils and can be used in foods and the like as dispersed in fats and oils to easily supply β-glucans having bioregulatory functions and which is uniformly dispersible throughout a food to improve the taste, texture, etc. of the food and to facilitate development of flavor of the food.

Background Art:

[0002] β-Glucans are material attracting attention for applications because of their excellent bioregulatory functions that have recently been analyzed, such as lipid metabolism improving action, intestinal regulatory action, and blood sugar controlling action. Broad application of such material to processed foods will bring extreme benefits, not only contributing to enhancement of functionality of processed foods (addition of value) but matching the expectation of contribution to public health maintenance. β-Glucans are contained in gramineous plants. β-Glucan occurring in gramineous plants, for example, barley and oats are components making cell walls of endosperms in seeds and are distributed almost throughout the seeds. A β-glucan content in barley flour is generally 3 to 10% by weight, while varying according to the parts or species. Structurally, β-glucans are glucose polymers mainly constructed through 1-3-β- and 1-4-β-D-glucopyranose bonds.

[0003] JP 09 009 890 discloses the production of a fine particulate gel useful in food by using β-1,3-glucan. US 4,231,802 discloses a method of preparing powders and granules capable of forming a stabilized micro-dispersion of a difficult to disperse material in cold water. This method comprises mixing the difficult to disperse material in liquid form with a matrix comprising by weight from about 70 to about 99 parts of disintegrated β-1,4-glucan. WO 01 30359 discloses a cholesterol lowering and blood lipids lowering composition which can contain β-glucan. WO 99 25198 discloses foodstuffs and drink mixes containing extruded fiber-containing intermediates which can contain β-glucan. WO 94 21128 discloses fat-reduced laminated doughs which can contain β-glucan.

[0004] In manufacturing processed foods added with or enriched in β-glucans, for example, foods added with or enriched in barley β-glucans, conceivable methods of fortification include (1) addition of barley grains or flour and (2) addition of β-glucans extracted from barley bran or grains. According to the method (1), it is relatively easy to fortify with β-glucans by adding barley grains or barley flour to a raw material mix which mainly comprises wheat flour and has been used in the manufacture of confectionery products. Nevertheless the method is problematical in that the β-glucan content in the food that can be reached by fortification is limited since the β-glucan content in the added barley grains or flour is about 10% by weight at most and that such addition of barley grains or flour can impair the taste or texture or cause uneven baking thereby to reduce product values. Addition of barley bran that contains β-glucans and other functional components in larger proportions may be conceivable. However, addition of bran is more problematical, easily causing reduction of taste and texture and uneven baking, which results in reduced commercial value. Besides, where added to liquid substances such as fats and oils, barley grains, barley flour and barley bran are difficult to mix and disperse uniformly. It is very difficult to prepare eatable fat and oil compositions containing these materials.

[0005] To the contrary, the method (2) which utilizes extracted β-glucans is useful and advantageous in that a β-glucan content in a processed food is arbitrarily adjustable. However, if barley β-glucans, which have high water absorption, is added as such to a raw material mix mainly comprising wheat flour and kneaded with water, they easily form lumps. Such lumps make the mix non-uniform, leading to reduction of quality in taste and texture. Although it is possible to obtain foods having β-glucans dispersed therein relatively uniformly by adding barley β-glucans as previously dissolved in water to a raw material mix (mostly powdered), this method has poor workability and impracticability in the site of manufacture because it is not easy to prepare a uniform aqueous solution. That is, it takes time to dissolve, and the resulting aqueous solution is viscous. In the manufacture of foods added with extract containing β-glucans obtained from gramineous plants such as barley, it has therefore been awaited to establish a process for uniformly dispersing β-glucans in a food to conveniently provide a processed food or to develop such β-glucan materials originated in gramineous plants.

[0006] On the other hand, quality modifiers have been in use for a variety of purposes, such as improvement of taste or texture of foods, processing capabilities, cooking capabilities, and workability in food production steps.

[0007] For instance, in the production of bakery product dough it is a practice to use an agent for modifying stickiness, softness or hardness of dough to improve workability in dough preparation or shaping. For baking, too, it is required to use modifiers contributory to improvements on product values, such as improvement on volume and springiness and elimination of a bumpy rough surface developed on the baked products. For preservation or storage of bakery products, it is a subject to prevent or retard hardening and deterioration of physical properties, such as texture, with time after

baking, and modifiers have been developed to address the subject. For frozen dough, modifiers are indispensable additives for imparting freeze resistance to dough. To meet these purposes, various food additives have been used, including emulsifiers, such as polyglycerol fatty acid esters, fatty acid monoglycerides, and lecithin; oxidizing agents acting to enhance gluten network; enzymes, such as α-amylase and protease serving to modify gluten network; fats and oils containing the components recited above; saccharides, such as sorbitol and trehalose; thickening polysaccharides, such as xanthan gum and pectin; and gelatinized starch (α-starch).

[0008] Under these circumstances, today's consumers are highly concerned about safety while pursuing good taste and texture. There is a visible tendency to avoid synthetic additives and seek naturally-occurring substances. It has therefore been keenly desired to establish methods for settling the problems associated with foods by minimizing the amounts of the above-recited additives that may impair taste or texture or hinder manifestation of flavor, particularly synthetic emulsifiers but by using, if possible, only naturally-occurring substances; and to develop additives based on naturally-occurring substances.

[0009] Accordingly, an object of the present invention is to provide a β-glucan-containing fat and oil composition which is capable of supplying β-glucans having excellent bioregulatory functionality without impairing taste and texture.

Disclosure of the Invention:

[0010] The present invention provides a β-glucan-containing fat and oil composition characterized by containing β-glucans extracted from a gramineous plant.

[0011] The present invention provides the β-glucan-containing fat and oil composition which contains a β-glucan having at least two kinds of bonds selected from a 1-2-β-D-glucopyranose bond, a 1-3-β-D-glucopyranose bond, a 1-4-β-D-glucopyranose bond, and a 1-6-β-D-glucopyranose bond.

[0012] The present invention provides the β-glucan-containing fat and oil composition which contains a β-glucan having a 1-3-β-D-glucopyranose bond and a 1-4-β-D-glucopyranose bond.

[0013] The present invention provides the β-glucan-containing fat and oil composition, wherein the gramineous plant is barley or oats.

[0014] The present invention provides a food containing the β-glucan-containing fat and oil composition.

[0015] The present invention provides a fat and oil emulsified composition containing the β-glucan-containing fat and oil composition.

[0016] The present invention provides a bakery product containing the β-glucan-containing fat and oil composition.

[0017] The present invention provides a confectionery product containing the β-glucan-containing fat and oil composition.

[0018] The present invention provides a food having a prophylactic action for habitual diseases (or life style-related diseases) containing the β-glucan-containing fat and oil composition.

[0019] The present invention provides a drug having a prophylactic action for habitual diseases containing the β-glucan-containing fat and oil composition.

[0020] The present invention provides a processed rice, wheat, maize or soybean product containing the β-glucan-containing fat and oil composition.

[0021] The present invention provides a liquid food containing the β-glucan-containing fat and oil composition.

[0022] The present invention provides a processed food mainly comprising starch and containing the β-glucan-containing fat and oil composition.

Best Mode for Carrying out the Invention:

[0023] The present invention will be described hereunder in detail. β-Glucans extracted from gramineous plants (hereinafter also referred to as "extracted β-glucans" or "β-glucan extract") which can be used in the fat and oil composition according to the present invention are not particularly restricted in the manner of extraction. An extracting solvent is added to a gramineous plant as a feed for extraction, and the plant is extracted. Any extract can be used irrespective of the extracting method, form or purity. For example, an extract layer *per se* obtained by solid-liquid separation, a liquid or solid β-glucan concentrate obtained from the extract layer in a conventional manner, and a liquid or solid with increased purity prepared from the extract layer with a conventional purification means are included. It is no problem if the extract contains extracted components other than β-glucans. In the present invention, all these substances are called "β-glucans extracted from a gramineous plant".

[0024] Gramineous plants include cereals, such as rices, wheats, maizes, sorghums, barnyard millets, foxtail millets, millets, barleys, oats, and ryes. While a whole plant can be used as a feed for extraction, seeds having relatively high β-glucan contents are preferred. Any of whole grain flour, bran by-produced in grain polishing, malt, germs, and endosperms can be used. Preferred feeds include whole grain flour of barleys or oats, endosperms separated from an outer part of grains of barleys or oats by polishing, brans generated from barleys or oats in polishing, rice bran, and

brans and germs separated form wheat or maize by polishing. Still preferred feeds are whole grain flour of barleys or oats, endosperms separated from an outer part of grains of barleys or oats by polishing, and brans generated from barleys or oats in polishing.

[0025] It is preferred for the β-glucans of the present invention extracted from gramineous plants to comprise a β-glucan having at least two kinds of bonds selected from a 1-2-β-D-glucopyranose bond, a 1-3-β-D-glucopyranose bond, a 1-4-β-D-glucopyranose bond, and a 1-6-β-D-glucopyranose bond. It is preferred for the β-glucans of the present invention to contain a β-glucan having a 1-3-β-D-glucopyranose bond and a 1-4-β-D-glucopyranose bond.

[0026] The method for extracting β-glucans of the present invention from gramineous plants is described below. The β-glucans according to the present invention are water-soluble polymers and capable of being dissolved in water. They are separated by extracting, for example, grain flour of a gramineous plant with 2 to 100 times as much solvent, such as water, warm water, hot water, a salt solution, an aqueous acid or alkali solution or an organic solvent, at an arbitrary temperature for an arbitrary period of time. The extraction system is subjected to solid-liquid separation to obtain β-glucans. β-Glucans obtained by extraction with water, warm water or hot water are preferred. Those extracted with warm water at 4 to 80°C are still preferred. An extraction accelerators and the like may be added in extracting.

[0027] More specifically, methods for obtaining high-molecular-weight β-glucans from barley, etc. include a method in which waxy barley is extracted with water (JP-B-4-11197), a method of obtaining β-glucans having a weight average molecular weight of 100,000 to 1,000,000 from barley or oats which comprises alkali extraction, neutralization, and precipitation in an alcohol (JP-B-6-83652), and a method of extracting β-glucans in which bran separated by polishing barley, etc. to a polishing yield of 82% or less is extracted with hot water at 80 to 90°C (JP-A-11-225706). Low-molecular-weight β-glucans obtained by conventional molecular weight reduction of the β-glucans obtained by these methods are also useful. Any known reactions for hydrolyzing polysaccharides is adoptable for molecular weight reduction of β-glucans. For example, it is known that water-soluble polysaccharides are hydrolyzable by heat and pressure application in the presence of an acid. This is made use of for molecular weight reduction of β-glucans. Molecular weight reduction making use of enzymatic hydrolysis is also effective. Useful enzymes include 1,3-β-glucanase. β-Glucans obtained by directly extracting a grain raw material by the method disclosed in WO98/13056, Japanese Patent Application No. 2000-287920, etc. are also usable. The extraction accelerators and the like described in Japanese Patent Application No. 2000-295660 can be used.

[0028] The β-glucans extracted from a gramineous plant which can be used in the present invention are high-molecular compounds. While β-glucans having any weight average molecular weight are usable, those having a molecular weight of less than 3,000,000, preferably less than 500,000, still preferably less than 100,000, are suitable because the compatibility with fat and oil increases as the molecular weight decreases. The molecular weight of extracted β-glucans may be reduced in a conventional manner to have improved compatibility with fat and oil, or low-molecular β-glucans may be directly obtained by extraction.

[0029] The fats and oils or fat and oil compositions in which the β-glucans extracted from a gramineous plant are dispersed are not particularly limited and include soybean oil, rapeseed oil, mustard oil, cotton seed oil, safflower oil, sesame oil, corn oil, peanut oil, kapok oil, sunflower oil, rice oil, rice bran oil, coconut oil, palm oil, palm kernel oil, linseed oil, castor oil, olive oil, cocoa butter, tung oil, camellia oil, illippe butter, Borneo tallow, Mowrah, sal butter, borage oil, lauric acid fat and oil, hard butter, shea butter, oleic acid-linoleic acid fat and oil, erucic acid oil, linolenic acid oil, conjugate acid oil, oxyacid oil, Cuphea oil, crambe (*Crambe abyssinica*) seed oil, meadowfoam oil, lesquerella (*Lesquerella fendleri*) seed oil, macadamia oil, evening primrose (*Oenothera biennis* and *Oenothera lamarkiana*) seed oil, oil of seeds of borage (*Borago officinalis*) from the Boraginaceae family, oil of seeds of amaranth (*Amaranthus* L.; an annual plant of the Amaranthaceae family), pine seed oil, avocado oil, grapeseed oil, oil of dry microbial cells of the filamentous fungus *Mortierella alpina,* lipid of dry microbial cells of Labyrinthulae belong to Xanthophyta, lipid of dry microbial cells of *Schizochytrium* sp. belonging to Labyrinthulomycota, lipid of *Crypthecodinium cohnii,* butter lipid, ghee butter, milk fat, beef tallow, lard, sheep tallow, fats and oils of other land animals, fish oil, whale oil, codliver oil, fats and oils of other marine animals, short chain fatty acid-containing fats and oils, middle chain fatty acid-containing fats and oils, γ-oryzanol, γ-linolenic acid-containing fats and oils, α-linolenic acid-containing fats and oils, docosahexaenoic acid (DHA)-containing fats and oils, eicosapentaenoic acid (EPA)-containing fats and oils, plant sterol-containing fats and oils, trans acid-containing fats and oils, hydroxy acid-containing fats and oils, conjugated fatty acid-containing fats and oils, polyphenol-containing fats and oils, phospholipid-containing fats and oils, sphingolipids, tocotrienol-containing fats and oils, sitostanol fatty acid-containing fats and oils, n-3 polyunsaturated fatty acid-containing fats and oils, diglycerides, and other vegetable or animal fats and oils; processed oils derived from these fats and oils according to necessity, such as hydrogenated oils, slightly hydrogenated oils, hydrogenation isomerized oils, interesterified oils, and fractionated oils, fats and oils processed by two or more of these processes, and mixtures of two or more of these processed fats and oils. Additionally, disperse systems using these fats and oils as a dispersing medium or a dispersoid, such as emulsions (including W/O emulsions, O/W emulsions, double emulsions, i.e., O/W/O emulsions and W/O/W emulsions, and more complex multiple emulsions) and suspensions are also useful (such disperse systems will hereinafter be included under the category "fats and oils"). Further, foods containing the above-recited fats and oils are also designated "fat and oil compositions" in the

present invention.

[0030] When added to fats and oils, the form of β-glucans extracted from a gramineous plant is not particularly limited and can be added as such or as dissolved in water or any other water-soluble solvent. In preparing β-glucan-containing emulsions, β-glucans may be dispersed in a previously prepared fat and oil emulsion or be dispersed at the time of emulsification. Addition of β-glucans can be carried out with no difficulty nor fail whether the fat and oil is liquid oil or solid fat.

[0031] The fat and oil composition containing β-glucans extracted from a gramineous plant is obtained by adding β-glucans extracted from a gramineous plant to fat and oil or a fat and oil composition, followed by mixing. Mixing means are not particularly limited, and mixing devices, such as a mixer, can be used. In particular, mixing β-glucans extracted from a gramineous plant with fat and oil or a fat and oil composition and keeping the mixture at 50°C or higher for a given time, preferably 5 minutes to 6 hours, still preferably 10 minutes to 2 hours, give a fat and oil composition in which the β-glucans extracted from a gramineous plant is uniformly or thoroughly compatible with the fat and oil. In foods produced by using the β-glucans extracted from a gramineous plant which are in a thoroughly compatible state with fat and oil, the β-glucans of a gramineous plant origin are dispersed more uniformly than in foods produced by directly adding β-glucans extracted from a gramineous plant. As a result, there are produced remarkable effects such that the taste or texture is not impaired, reduction of flavor due to an emulsifier is unexpectedly suppressed, and manifestation of the flavor of a food is improved.

[0032] It is a feature of the fat and oil composition of the present invention that β-glucans are very uniformly dispersed in fat and oil by use of various mixing, kneading or stirring machines described later.

[0033] Where the fat and oil composition is composed of an oily phase and an aqueous phase as with an emulsion, while it is possible to add β-glucans extracted from a gramineous plant to either the oily phase or the aqueous phase, it is preferred that the extracted components of gramineous plant origin be first dispersed completely in the oily phase and then mixed with the aqueous phase so that the β-glucans extracted from a gramineous plant may exhibit satisfactory compatibility with fat and oil to give a homogeneous fat and oil composition. Thus, a fat and oil composition containing β-glucans extracted from a gramineous plant can be obtained in a short time.

[0034] Where β-glucans extracted from a gramineous plant are incorporated into a water-in-oil emulsion, a plasticized water-in-oil emulsion, etc., addition of β-glucans extracted from a gramineous plant may be followed by emulsification, or be simultaneous with emulsification, or be preceded by emulsification as stated above, or be preceded by plasticization. In using solid fat, it may be softened or liquefied by an appropriate method according to necessity, and β-glucans extracted from a gramineous plant is then added thereto. In order to highly uniformly disperse β-glucans extracted from a gramineous plant, it is desirable that 100 parts by weight of powdered β-glucans and 10 to 50 parts by weight of edible fat and oil are mixed, and the mixture is then subjected to rolling or a combination of rolling and conching. Other raw materials, an additional amount of oil, and the like may be added in this state to adjust the β-glucan content in the finally obtained fat and oil composition.

[0035] The means for mixing β-glucans extracted from a gramineous plant into fats and oils include various types of machines for mixing, kneading or stirring. Examples are propeller agitators, oscillatory mixers, orifice mixers, paddle agitators, agitation emulsifiers (homomixer), cutter mixers, cokneaders, conches, silent cutters, jet mixers, vacuum agitators, screw mixers, static mixers, cutting mixers, sonicators, kneaders, rolls, Hydrossure, pipeline mixers, universal mixers, pin machines, homogenizers (high-pressure homogenizers), ball cutters, and ribbon mixers. It is preferred to use an agitation emulsifier (homomixer) and/or a homogenizer (high-pressure homogenizer) at a product temperature of 40° to 80°C. After mixing by agitation, the fat and oil composition containing β-glucans extracted from a gramineous plant may be stored as obtained, or emulsified, or rapidly cooled for plasticization. For plasticization, a votator, a combinator, a perfector, a complector, Onreitor etc. can be used. Use of a pin machine at a product temperature of 10°C or lower is preferred. It is also possible that β-glucans extracted from a gramineous plant is added to fat and oil having been emulsified and then processed in a rapid cooling-plasticizing apparatus, such as a votator, a combinator, a perfector, a complector or a scraped-surface heat exchanger, and the mixture is treated by any of the above-described methods to prepare a fat and oil composition containing β-glucans extracted from a gramineous plant.

[0036] The content of β-glucans extracted from a gramineous plant in the fat and oil composition of the present invention is desirably 0.01 to 500 parts by weight, preferably 0.1 to 150 parts by weight, still preferably 1 to 100 parts by weight, per 100 parts by weight of the total composition except the β-glucans. Where the content of β-glucans extracted from a gramineous plant is less than 0.01 part by weight, a final product tends to fail to exhibit the functional effects of the β-glucans. If it exceeds 500 parts by weight, the mixture tends to become powdery or lumpy irrespective of the kinds of other ingredients, failing to provide an edible fat and oil composition having β-glucans extracted from a gramineous plant uniformly mixed and dispersed therein. Even after the mixture is processed into a final product, the lumps would remain only to cause non-uniform distribution of the extract.

[0037] Where an extract layer obtained by extracting a gramineous plant is used as such without being purified or merely after being powderized or solidified, the β-glucan purity of the extract is preferably as high as possible. An acceptable purity ranges 1 to 100%, and a preferred purity is from 10 to 100%, particularly 20 to 100%.

[0038] It is possible to add, to the fat and oil composition containing β-glucans extracted from a gramineous plant of

the present invention, food additives or foods such as emulsifiers, gelling agents, thickeners, and stabilizers to prevent the β-glucans from getting distributed non-uniformly due to agglomeration into lumps in the composition. The foods or food additives are not particularly limited as far as they are edible. Examples of the emulsifiers are lecithin, fatty acid monoglycerides, sorbitan fatty acid esters, propylene glycol fatty acid esters, sugar esters, polyoxyethylene sorbitan fatty acid esters, Tween, polyoxyethylene sorbitan trioleate, polyoxyethylene sorbitan monooleate, polyoxyethylene sorbitan monostearate, polyoxyethylene sorbitan monopalmitate, and polyoxyethylene sorbitan monolaurate. Examples of the thickeners and the stabilizers are pullulan, psyllium, gum arabic, gellan gum, glucomannan, guar gum, xanthan gum, tamarind gum, carrageenan, alginic acid salts, farceran, locust bean gum, pectin, curdlan, and low-molecular compounds obtained from these substances, starch, processed starch, gelatinized starch, crystalline cellulose, gelatin, dextrin, agar, and dextran. Additional useful food additives or foods include saccharides, such as glucose, fructose, sucrose, maltose, enzyme-saccharified sugar (thick malt syrup), lactose, reducing starch saccharification products, isomerized liquid sugar, sucrose-coupled malt syrup, oligosaccharides, reducing sugar polydextrose, sorbitol, reduced lactose, trehalose, xylose, xylitol, maltitol, erythritol, mannitol, fructo-oligosaccharides, soybean oligosaccharides, galacto-oligosaccharides, lactosucrose-oligosaccharides, raffinose, lactulose, palatinose-oligosaccharides, stevia, and Aspartame; stabilizers, such as phosphoric acid salts (e.g., hexametaphosphorates, secondary phosphates and primary phosphates) and alkali metal (e.g., potassium or sodium) salts of citric acid; proteins, such as whey proteins, (e.g., α-lactalbumin, β-lactoglobulin, and serum albumin), casein and other milk proteins, low-density lipoprotein, high-density lipoprotein, egg proteins (e.g., phosvitin, livetin, phosphoglycoprotein, ovalbumin, conalbumin, and ovomucoid), wheat proteins (e.g., gliadin, glutenin, prolamine, and glutelin), and other vegetable and animal proteins; inorganic salts, such as sodium chloride, rock salt, sea salt, and potassium chloride; souring agents, such as acetic acid, lactic acid, and gluconic acid; colorants, such as β-carotin, caramel, and Monascus color; antioxidants, such as tocopherol and tea extract; eggs, such as whole eggs, egg yolk, egg white, and enzyme-processed eggs; cereals, such as bread flour, all-purpose flour, and cake flour; beans, such as soybean powder; water, flavors, dairy products, seasonings, pH adjustors, enzymes, food preservatives, shelf life extenders, fruits, fruit juices, coffee, nut pastes, spices, cacao mass, and cocoa powder. Two or more of these additives can be used in combination. The amounts of the additives to be added are not particularly limited. They can be added in general amounts, for example, 0.01 to 15% by weight based on the composition.

[0039] The foods to which the fat and oil composition of the present invention is applicable will then be described. The foods that can be used are those containing the aforementioned fat and oil composition containing β-glucans extracted from a gramineous plant as a part or the whole of the fats and oils conventionally employed therefor or those containing β-glucans extracted from a gramineous plant and fats and oils. Included in such foods are not only fat and oil foods exemplified by margarine and shortening but any kinds containing fats and oils, such as bakery products, confectionery products, processed rice products, processed wheat products, processed maize products, processed soybean products, health foods, and medicinal foods. The fat and oil composition containing β-glucans extracted from a gramineous plant according to the present invention can be used as a substitute for a part of, or the whole of, a fat and oil composition used in these foods according to conventional usage whether it is liquid (e.g., salad oil, frying oil, and whipping cream), sol (e.g., liquid shortening), pasty or emulsion (e.g., foamable emulsified fats, dressings, fat spreads, custard cream, and dipping cream) or solid (e.g., shortening, margarine, candies, chocolates, and roux type curry sauce mixes).

[0040] Examples of products to which the fat and oil composition is applied include foods, food additives, cosmetics, toiletries, and drugs. The foods include grain-related products, such as those containing wheat flour as a main ingredient and those containing rice as a main ingredient, such as bread, dessert bread, hot dog buns, and Danish pies; pancakes, doughnuts, pizza, *tempura,* and premixes thereof; cookies/biscuits and snacks; noodles, such as raw noodles, dry noodles, packaged instant noodles, instant cup noodles, *udon* noodles (white wheat noodles), buckwheat noodles, Chinese noodles, rice noodles, and pastas; rice products, such as boiled rice, rice cake, sterile boiled rice, retort pouch boiled rice, nonglutinous rice flour, glutinous rice flour, dumplings, rice crackers, and rice chips; and toppings.

[0041] The foods also include confectionery products, either Japanese or European, such as chocolates, candies, drops, chewing gums, baked confections, cakes, and sweet bean-jam buns.

[0042] The foods also include processed meat products, such as ham, sausage, and hamburgs; and processed marine products, such as steamed fish paste, baked fish paste, fried fish paste, and fish sausage.

[0043] The foods also include dairy products, such as butter, cheese, ice cream, and yogurt.

[0044] The foods also include beverages, such as alcoholic beverages, e.g., beer, *sake,* whisky, brandy, *sho-chu* (Japanese distilled liquor), distilled liquors, sparkling alcohol, wines, and fruit wines; coffee, tea, green tea, woolong tea, Chinese tea, cocoa, carbonated beverages, nutritious drinks, sports drinks, coffee drinks, lactic acid beverages, fruit juices, and fruit drinks.

[0045] The foods also include processed fat and oil products, such as margarine, shortening, mayonnaise, and cream.

[0046] The foods also include seasonings and sauces, such as spices, dips, dressings for meat, salad dressings, Worcester sauce, *miso,* soy sauce, sauce mixes, e.g., curry sauce mixes and hashed beef sauce mixes; soups, such as corn soup, potato soup, and pumpkin soup; jams, peanut butter; and toppings.

[0047] The foods also include preserved foods, such as canned or bottled foods of fishes and shells, meats, fruits,

vegetables, mushrooms, corned beef, jams, tomatoes, etc.; frozen foods; retort pouch foods, such as curry, stew, meat sauce, ma-po tofu, stew of meat with vegetables, soups, and boiled rice; and powdered foods, such as instant powder foods, e.g., powdered beverages, powdered soups, and powdered *miso* soup.

[0048] The foods also include special health foods, such as baby foods (e.g., weaning foods), invalid diets (e.g., liquid diets), diets for the old, fat-reducing diets, and supplementary foods.

[0049] The foods also include microwave foods ready to be reheated or cooked.

[0050] The foods also include staple diets such as bread, noodles, and rice.

[0051] The foods also include those easy to take everyday or regularly.

[0052] The foods also include those easy to add to other foods.

[0053] The foods also include those which tend to be insufficient, those necessary for holding biobalance, and those which, when combined with other foods, result in better nutriment.

[0054] The foods also include those, when combined with other foods, result in a synergetic effect.

[0055] The fat and oil emulsified composition will be described in more detail.

[0056] The fat and oil emulsified composition according to the present invention is not particularly limited as long as it comprises fat and oil, water, and β-glucans extracted from a gramineous plant.

[0057] The extracted β-glucan content in the fat and oil emulsified composition is preferably 0.005 to 20% by weight, still preferably 0.5 to 5% by weight.

[0058] The fat and oil emulsified composition includes oil-in-water (O/W) emulsions, water-in-oil (W/O) emulsions, and double emulsions, i.e., O/W/O emulsions and W/O/W emulsions, and more complex multiple emulsions. Specific examples of these emulsions are margarine, fat spread, butter cream, custard cream, flour paste, whipped cream, mayonnaise, dressings, white sauce, tartar sauce, milk creams (mainly comprising raw milk, milk or a residue after removing components other than milk fat from milk) used in ice cream, cakes, etc. or with coffee, and cooking creams (raw cream, creme chantily or creme fouette, cream for entremets, cream for patisserie, butter cream, creme patissiere, creme anglaise, and creme Saint Honore). These fat and oil emulsified compositions are obtained with excellent emulsion stability, flavor, and texture. In the case of sour oil-in-water emulsions such as mayonnaise, the effect of softening sourness is manifested to provide a natural vinegar flavor.

[0059] The amount of fat and oil is not particularly limited and decided according to the type of the emulsified compositions. For example, the fat and oil content of oil-in-water emulsified compositions is preferably 4 to 85% by weight, still preferably 4 to 70% by weight, particularly preferably 4 to 50% by weight, and that of water-in-oil emulsified compositions is preferably 40 to 95% by weight, still preferably 45 to 90% by weight, particularly preferably 50 to 80% by weight.

[0060] The water content is not particularly limited and decided according to the type of the emulsified compositions. For example, the water content in oil-in-water emulsified compositions is preferably 15 to 95% by weight, still preferably 30 to 95% by weight, and that in water-in-oil emulsified compositions is preferably 5 to 45% by weight, still preferably 10 to 30% by weight.

[0061] The fat and oil emulsified compositions containing β-glucans extracted from a gramineous plant are prepared by adding β-glucans to the aqueous phase and/or the oily phase, and the aqueous phase and the oily phase are emulsified in a usual manner.

[0062] If desired, the resulting fat and oil emulsified composition of the present invention may be homogenized by means of a homogenizing machine, such as a valve homogenizer, a homomixer, and a colloid mill. If desired, the fat and oil emulsified composition may be subjected to pasteurization or heat sterilization treatments, such as UHT treatments by a direct heating system (e.g., injection or infusion) or an indirect heating system by use of a plate, tubular or scraped-surface heat exchanger, HTST treatments, a batch treatment, a retort treatment, and microwave heating, or cooking on the fire. According to necessity, the composition may be subjected to re-homogenization. If necessary, the composition may be subjected to a cooling operation such as rapid cooling or slow cooling. If necessary, the composition may be subjected to aging. Further, the fat and oil emulsified composition thus obtained may be refrigerated or frozen for storage, if needed.

[0063] In preparing the fat and oil emulsified composition of the present invention, emulsification, homogenization, mixing or preparation of raw materials can be carried out with mixing, kneading or stirring equipment of various kinds commonly employed with no particular restriction. Examples are propeller agitators, oscillatory mixers, orifice mixers, paddle agitators, agitation emulsifiers (homomixer), cutter mixers, cokneaders, conches, silent cutters, jet mixers, vacuum agitators, screw mixers, static mixers, cutting mixers, sonicators, kneaders, rolls, Hydrossure, pipeline mixers, universal mixers, pin machines, colloid mills, homogenizers (high-pressure homogenizers), jet homogenizers, ball cutters, and ribbon mixers. It is preferred to use an agitation emulsifier (homomixer) and/or a homogenizer (high-pressure homogenizer), a jet homogenizer or a colloid mill. Equipment used for heating or cooling includes indirect heating systems using a scraped surface heat exchanger, e.g., Contherm scraped surface heat exchanger (manufactured by Tetra Laval Food), Thermoclinder (manufactured by Iwai Kikai), a surface scraped heat exchanger (manufactured by Izumi Food Machinery Co., Ltd.), CP-Rotorpro scraped surface heat exchanger (manufactured by APV) or Terlotherm scraped surface heat exchanger (manufactured by Terlet), and steam injection direct heating systems, e.g., Steam Nozzle (manufactured by

Iwai Kikai), Noritake Cooker (manufactured by Noritake Company), and Steam Injection (manufactured by Tetra Pack).

[0064] The fat and oil emulsified composition of the present invention exhibits excellent emulsion stability without using conventional emulsifiers, particularly synthetic emulsifiers, and resistance to freezing or microwave cooking and has good taste and texture and, in addition, a bioregulatory function. As a matter of course, the composition may contain other components effective on emulsification and emulsion stabilization, such as natural emulsifiers, enzymatically treated substances, and thickening polysaccharides, e.g., substances originated in eggs, milk, soybeans or gramineous cereals or enzyme-treated substances thereof.

[0065] Examples of such natural emulsifiers or enzymatically treated substances which can be used as an emulsifier include lecithins (e.g., soybean lecithin and egg yolk lecithin), eggs (whole eggs, egg white or egg yolk), milk, ground or dried soybeans, flours of grains, such as wheat and barley, proteins separated from these food materials such as egg protein, defatted milk powder, milk protein, whey proteins, (e.g., α-lactalbumin, β-lactoglobulin, and serum albumin), milk serum protein, casein, sodium casein, and other milk proteins, egg white albumin, ovomucoid, low-density lipoprotein, high-density lipoprotein, egg proteins (e.g., phosvitin, livetin, phosphoglycoprotein, conalbumin, and ovomucoid), proteins of grain origin, such as wheat proteins (e.g., gluten and gliadin), zein, glutenin, and prolamine, soybean protein isolate, and other vegetable and animal proteins, protein-polysaccharide complexes, and substances left after removing components other than milk fat from raw milk, processed milk, concentrated milk, raw sheep milk, etc. Enzyme-treated substances obtained by contact reaction between these materials with enzymes, for example, soybean protein hydrolyzate, wheat protein hydrolyzate, milk protein hydrolyzate, and egg protein hydrolyzate, are also useful. One or more of these substances can be used in combination. The amount of these substances to be added is not particularly limited and can be varied appropriately. They are added in generally adopted amounts, for example, 0.01 to 15% by weight based on the composition of the present invention.

[0066] Any enzyme that is expected to modify the materials such as eggs, milk, soybeans and grains to improve emulsifying capabilities can be used to obtain the above-mentioned enzyme-treated substances. Such enzymes include amylases, e.g., α-amylase, β-amylase, and glucoamylase), cellulases (e.g., cellulase and hemicellulase), proteases, lipases (e.g., lipase and phospholipase), glucose oxidase, lipoxygenase, lactase, invertase, pentonase, pectinase, catalase, ascorbic acid oxidase, sulfhydryl oxidase, and hexose oxidase. The term "amylase" denotes one or more amylases selected from the group consisting of α-amylase, isoamylase, and glucoamylase. Amylases produced by microorganisms belonging to the genera Bacillus, Pseudomonas, Aspergillus, Rhizopus and Klebsiella are preferred.

[0067] Flour of grains includes crude or refined flour of rices, wheats, maizes, sorghums, barnyard millets, foxtail millets, millets, barleys, oats, and ryes. Wheat flour includes bread flour, less strong bread flour, allpurpose flour, cake flour, and duram flour. Buckwheat flour, soybean flour are also usable.

[0068] The thickening polysaccharides include pullulan, psyllium, gum arabic, gellan gum, glucomannan, guar gum, xanthan gum, tamarind gum, carrageenan, alginic acid alkali metal salts, farceran, locust bean gum, pectin, curdlan, crystalline cellulose, CMC, gelatin, dextrin, agar, dextran, and low-molecular compounds obtained therefrom. These polysaccharides may be used either individually or as a combination of two or more thereof. A preferred content of the thickening polysaccharides is 0.1 to 5% by weight, particularly 0.5 to 3% by weight. With a thickening polysaccharide content less than 0.1% by weight, the composition tends to have poor shape retention. With contents exceeding 5% by weight, the composition tends to be hard and hardly melt in the mouth.

[0069] It is possible to add, to the fat and oil emulsified composition of the present invention, food additives or foods such as emulsifiers, gelling agents, thickeners, and stabilizers other than those described above as long as the effects of the present invention are not impaired. The foods or food additives are not particularly limited as far as they are edible. Examples of the emulsifiers are fatty acid monoglycerides, sorbitan fatty acid esters, propylene glycol fatty acid esters, sugar esters, polyoxyethylene sorbitan fatty acid esters, Tween, polyoxyethylene sorbitan trioleate, polyoxyethylene sorbitan monooleate, polyoxyethylene sorbitan monostearate, polyoxyethylene sorbitan monopalminate, and polyoxyethylene sorbitan monolaurate. Examples of the thickeners and stabilizers are starch, modified starch, and gelatinized starch. Additional useful food additives or foods include saccharides, such as glucose, fructose, sucrose, maltose, enzyme-saccharified sugar (thick malt syrup), lactose, reducing starch saccharification products, isomerized liquid sugar, sucrose-coupled malt syrup, oligosaccharides, reducing sugar polydextrose, sorbitol, reduced lactose, trehalose, xylose, xylitol, maltitol, erythritol, mannitol, fructo-oligosaccharides, soybean oligosaccharides, galacto-oligosaccharides, lactosucrose, raffinose, lactulose, palatinose-oligosaccharides, stevia, and Aspartame; stabilizers, such as phosphoric acid salts (e.g., hexametaphosphoates, secondary phosphates and primary phosphates) and alkali metal (e.g., potassium or sodium) salts of citric acid; inorganic salts, such as sodium chloride, rock salt, sea salt, and potassium chloride; souring agents, such as acetic acid, lactic acid, gluconic acid, citric acid, succinic acid, malic acid, and ascorbic acid; colorants, such as β-carotin, caramel, and Monascus color; antioxidants, such as tocopherol and tea extract; flavors, such as milk flavor, vanilla flavor, vanilla essence, chocolate flavor, strawberry flavor, and cheese flavor; dairy products, seasonings, pH adjustors, enzymes, food preservatives, shelf life extenders, fruits, fruit juices, coffee, nut pastes, spices, cacao mass, and cocoa powder. Two or more of these additives can be used in combination. The amounts of the additives to be added are not particularly limited. They can be added in general amounts, for example, 0.01 to 15% by weight based

on the composition of the present invention.

[0070] It is particularly preferred to add tocopherol to improve antioxidation stability of the fat and oil emulsified composition.

[0071] Application of the fat and oil compositions to bakery products will then be described. The bakery products contain the aforementioned fat and oil composition containing β-glucans extracted from a gramineous plant. The bakery products include those produced by (a) preparing dough by mixing the fat and oil composition as a part or the whole of conventional fat and oil with water and, in addition, secondary raw materials suited to the product, such as fats and oils, emulsions, sugars, dairy products, eggs, yeast, yeast food, oxidizing agents, reducing agents, various enzymes, and emulsifiers, which are added either all at once or in divided portions and (b) subjecting the resulting dough, either as prepared or after fermentation, to a heating treatment, such as steaming, baking, frying, and boiling, or (b') preserving the resulting dough by refrigeration or freezing and then (b) subjecting the dough to a heating treatment, such as steaming, baking, frying, and boiling. The bakery products include breads (e.g., pancakes, crepe, sponge cakes, doughnuts, dessert bread, loaves of bread, hot dog buns, Danish pies, French bread, rye bread, hard rolls, and Danish pastries), pies, *kasutera* (Japanese style sponge cake), butter cakes, puff pastries, waffles, biscuits/cookies, crackers, and fermented confections. The method of preparing dough is not particularly limited, except that a part of or the whole of the fat and oil which has been conventionally used in dough preparation is substituted with the fat and oil composition containing β-glucans extracted from a gramineous plant according to the present invention. In making bread as an example of the bakery products, bread dough is prepared from general raw materials of bread, such as wheat flour, water, yeast, sugar, and edible salt, and the edible fat and oil composition containing β-glucans extracted from a gramineous plant by a known dough preparation method. For instance, after mixing the other materials, the fat and oil composition containing β-glucans extracted from a gramineous plant may be folded into the mix. The dough is then fermented, shaped, and baked in a usual manner. Similarly, the fat and oil composition containing β-glucans extracted from a gramineous plant can be used as a substitute for a part of or the whole of fat and oil for folding (roll-in fat and oil) or fat and oil for dough and batter in making folded pies; or pieces of fat and oil in the form of chips, straws, etc. in making pie crusts; or foamable emulsified fat and oil or liquid oil for cakes in making sponge cakes.

[0072] Where the production of bakery products involves a baking step, if barley grains or barley flour is added as such as a β-glucan supply source or even when β-glucans extracted from a gramineous plant is added to dough or a powder mix, which is then kneaded into dough, lumps are easily formed in the dough. Dough having such lumps only provides a bakery product with a rough or grainy texture or a strange texture due to non-uniformity of the water content or hardness. To the contrary, use of the fat and oil composition containing β-glucans extracted from a gramineous plant provides dough having the β-glucans extracted from a gramineous plant uniformly dispersed therein with very few lumps, which finally gives a baked product with a good texture, not only free from a strange texture but with greatly improved softness. The fat and oil composition of the present invention is preferably used in an amount of 0.05 to 30% by weight, in terms of the β-glucans extracted from gramineous plants, based on the grain flour content of the bakery product.

[0073] In using the fat and oil composition in the bakery products, the bakery products can additionally contain at least one additive selected from the group consisting of an emulsifier, an oxidizing agent, and an enzyme and a glucide having at least one of an 1-2-α-D-glucopyranose bond, an 1-3-α-D-glucopyranose bond, an 1-4-α-D-glucopyranose bond, and an 1-6-α-D-glucopyranose bond, D-fructose or a saccharide containing D-fructose.

[0074] The emulsifier is preferably at least one selected from fatty acid monoglycerides, diacetyltartaric esters, and sucrose fatty acid esters. Still preferred are diacetyltartaric esters, such as diacetyltartaric acid monoglyceride and diacetyltartaric acid diglyceride. Lecithin, sorbitan fatty acid esters, propylene glycol fatty acid esters, sugar esters, and the like are also useful as the emulsifier.

[0075] The emulsifier is preferably used in an amount of 1 to 300 parts by weight per 100 parts by weight of the β-glucans extracted from a gramineous plant and 0.01 to 5% by weight, particularly 0.08 to 1% by weight, based on the grain flour in the bakery product.

[0076] The diacetyltartaric acid monoglyceride, which is preferably used as the emulsifier, is not particularly limited by the process of preparation and is usually obtained by reacting diacetyltartaric anhydride with a mixture of a monoglyceride and a diglyceride in the presence of acetic acid or esterifying a mixture of a monoglyceride and a diglyceride with tartaric acid and acetic acid in the presence of acetic anhydride. Accordingly, the diacetyltartaric acid monoglyceride which is preferably used in the present invention is usually obtained as containing diacetyltartaric acid diglyceride. Diacetyltartaric acid monoglyceride in an aqueous solution or an oil-in-water emulsion has a pH ranging from about 1.3 to 1.9. The composition used in the bakery products of the present invention preferably has its pH adjusted within 3.0 to 10.0, particularly 4.5 to 8.5, to improve stability. If necessary, the pH is adjusted by addition of a base or a salt having a pH adjusting action. Any base or salt that has a pH adjusting action and is permitted to be added to foods can be used. Preferred bases or salts include such bases as ammonia, calcium hydroxide, sodium hydroxide, and potassium hydroxides; sodium, potassium, ammonium or calcium salts of acetic acid, carbonic acid, phosphoric acid, succinic acid, glutamic acid, ascorbic acid, tartaric acid, etc.; and sodium hydrogencarbonate.

[0077] The oxidizing agent which can preferably be used in the bakery products is at least one selected from cystine

and ascorbic acid. Ascorbic acid is still preferred.

[0078] The oxidizing agent is preferably used in an amount of 0.01 to 15 parts by weight per 100 parts by weight of the β-glucans extracted from a gramineous plant and 0.0001 to 0.2% by weight, particularly 0.0005 to 0.1% by weight, based on the grain flour in the bakery product.

[0079] The enzymes which can be used in the bakery products include any kind having improving effects in bread-making. At least one enzyme selected from the group consisting of amylases, proteases, cellulases, and hemicellulases is preferably used. Other useful enzymes include lipases, glucose oxidase, lipoxigenase, lactase, invertase, pentonase, pectinase, catalase, ascorbic acid oxidase, sulfhydryl oxidase, hexose oxidase, and transglutaminase.

[0080] The enzyme content varies depending on the purity of enzyme preparations or the kind of the enzyme. In using a commercially available enzyme preparation, for example, an advisable amount is 0.01 to 100 parts by weight, preferably 0.05 to 30 parts by weight, per 100 parts by weight of the β glucans extracted from a gramineous plant.

[0081] The enzymes to be used will be described in greater detail. The term "amylase" denotes one or more amylases selected from the group consisting of α-amylase, isoamylase, and glucoamylase. Amylases produced by microorganisms belonging to the genera Bacillus, Pseudomonas, Aspergillus, Rhizopus and Klebsiella are preferred. Commercially available amylase preparations can be utilized. Commercially available α-amylase preparations include Amylase AD Amano and AH Amano from Amano Seiyaku K.K.; Termamyl and BAN from Novo Nordisk Bioindustry Ltd.; Uniase BM-8 from Yakult Honsha Co., Ltd.; Spitase HS, HK, PN-4, AI, and LH from Nagase Seikagaku Kogyo K.K.; Sumizyme L from Shin-Nihon Kagaku Kogyo K.K.; Kleistase from Daiwa Fine Chemicals Co., Ltd.; Rohalase AT from Röhm Enzyme GmbH; liquefied enzymes T, K, and SS from Ueda Kagaku K.K.; Biozyme C and L from Amano Seiyaku K.K., and Uniase L from Yakult Honsha Co., Ltd. Commercially available isoamylase preparations include Plullanase Amano and DB-1 from Amano Seiyaku K.K.; and Promozyme from Novo Nordisk Bioindustry Ltd. Commercially available glucoamylase preparations include Glucozyme NL and AF6 from Amano seiyaku K.K.; Sumizyme S, SG, AN, AD, and AL from Shin-Nihon Kagaku Kogyo K.K.; AMG and Dextrozyme from Novo Nordisk Industry Ltd.; Glucozyme from Nagase Seikagaku Kogyo K.K.; Uniase K from Yakult Pharmaceutical Ind. Co., Ltd.; Glutase 600 and AD from Hankyu Bioindustry K.K.; Kokugen and Daizyme from Daiwa Fine Chemicals Co., Ltd.; and Kokulase G2 and Sanzyme from Sankyo Co., Ltd. All these enzyme names are trade names.

[0082] The proteases can be of any origin, such as the genus Bacillus, Thermus or Aspergillus, plants, and animals. Preferred proteases are of the genus Bacillus, Thermus or Aspergillus or plants. The proteases can be used either individually or as a combination of two or more thereof. Commercially available protease preparations can be utilized. Examples of commercially available protease preparations are Protease N Amano, P Amano, and S Amano from Amano Seiyaku K.K.; Alkalase and Neutrase from Novo Nordisk Bioindustry Ltd.; Sumizyme LP from Shin-Nihon Kagaku Kogyo K.K.; and Thermoase from Daiwa Fine Chemicals Co., Ltd. All the enzyme names recited above are trade names. Pepsin, trypsin, chymotrypsin, papain, and chymosin (rennet) are also usable.

[0083] The cellulases preferably include those originated in the genus Trichoderma, Aspergillus or Fusarium. Commercially available various cellulase preparations can be made use of either individually or as a combination of two or more thereof. Examples of such preparations are Cellulase A and T from Amano Seiyaku K.K.; Sumizyme AC and C from Shin-Nihon Kagaku Kogyo K.K.; Cellulase XP and Celluzyme from Nagase Seikagaku Kogyo K.K.; Cellulase Onozuka and Y-NC from Yakult Pharmaceutical Ind. Co., Ltd.; Meicelase TP, TL and HP from Meiji Seika Kaisha, Ltd.; Driserase from Kyowa Hakko Kogyo Co., Ltd.; and GODO-TCL and TCL-H from Godo Shusei Co., Ltd. All the enzyme names recited above are trade names.

[0084] Commercially available various hemicellulase preparations can be used as the hemicellulase either individually or as a combination of two or more thereof. Examples of the hemicellulase preparations are Hemicellulase Amano from Amano Seiyaku K.K.; Sumizyme X from Shin-Nihon Kagaku Kogyo K.K.; Olivex from Novo Nordisk Bioindustry Ltd.; Cellulosin HC, B, TP25 and GM5 from Hankyu Bioindustry K.K.; and GODO-TXL from Godo Shusei Co., Ltd. All these enzyme names are trade names.

[0085] The lipases preferably include those of the genus Aspergillus, Rhizopus or Pseudomonas origin. Various commercially available lipase preparations can be used either individually or as a combination of two or more thereof. Such lipase preparations include Lipase A Amano or P Amano from Amano Seiyaku K.K.; and Lipase (Saiken) from Nagase Seikagaku Kogyo K.K. All these enzyme names are trade names.

[0086] The glucide having at least one of an 1-2-α-D-glucopyranose bond, an 1-3-α-D-glucopyranose bond, an 1-4-α-D-glucopyranose bond, and an 1-6-α-D-glucopyranose bond which can be used in the bakery products includes starch and glycogen, which are 1-4-α-glucans having an 1-4-α-D-glucopyranose bond; dextran, which is an 1-6-α-glucan having an 1-6-α-D-glucopyranose bond, and mucin which is an 1-3,1-6-α-glucan having an 1-3-α-D-glucopyranose bond and an 1-6-α-D-glucopyranose bond.

[0087] A preferred glucide content is such that the ratio of the β-glucans extracted from a gramineous plant to the glucide ranges from 1/1000 to 0.35/1 by weight.

[0088] The D-fructose or a saccharide containing D-fructose which can be used in the bakery products includes purified D-fructose, honey, sugar, isomerized sugar, and juices of pears, watermelons, etc.

[0089] A preferred content of D-fructose or the saccharide containing D-fructose is such that the ratio of the β-glucans extracted from a gramineous plant to the D-fructose or the saccharide containing D-fructose ranges from 1/40 to 15/1 by weight. With this amount, the dough is easy to work with, and bakery products with a good texture, softness, and good volume can be obtained. Even when frozen, the dough provides bakery products excellent in anti-staling properties. Where D-fructose or the saccharide containing D-fructose is used in an amount of 0.1 part by weight or more in terms of D-fructose per 100 parts by weight of the grain flour of the bakery product, the dough is easy to work with, and bakery products with a good texture, softness, and good volume can be obtained. Even when frozen, the dough provides bakery products excellent in anti-staling properties.

[0090] For use in bakery products, the fat and oil composition can contain various additives and vehicles.

[0091] Such additives and vehicles include food additives and foods, such as grain flour, yeast, yeast food, fat and oil, a fat and oil emulsified composition (e.g., a water-in-oil emulsion or an oil-in-water emulsion), wheat gluten, expanding agents (e.g., ammonium hydroge4ncarbonate and sodium hydrogencarbonate), bleaching agents (e.g., ammonium persulfate), water, an aqueous solution containing an alcohol, a polyhydric alcohol, a salt, an acid, an alkali, etc., gelling agents, thickeners, and stabilizers. These additives are not particularly limited as far as they are edible. For examples, fats and oils which can be added include soybean oil, rapeseed oil, mustard oil, cotton seed oil, safflower oil, sesame oil, corn oil, peanut oil, kapok oil, sunflower oil, rice oil, rice bran oil, coconut oil, palm oil, palm kernel oil, linseed oil, castor oil, olive oil, cocoa butter, tung oil, camellia oil, illippe butter, Borneo tallow, Mowrah, sal butter, borage oil, lauric acid fat and oil, hard butter, shea butter, oleic acid-linoleic acid fat and oil, erucic acid oil, linolenic acid oil, conjugate acid oil, oxyacid oil, Cuphea oil, crambe (*Crambe abyssinica*) seed oil, meadowfoam oil, lesquerella (*Lesquerella fendleri*) seed oil, macadamia oil, evening primrose (*Oenothera biennis* and *Oenothera lamarkiana*) seed oil, oil of seeds of borage (*Borago officinalis*) from the Boraginaceae family, oil of seeds of amaranth (*Amaranthus* L.; an annual plant of the Amaranthaceae family), pine seed oil, avocado oil, grapeseed oil, oil of dry microbial cells of the filamentous fungus *Mortierella alpina,* lipid of dry microbial cells of Labyrinthulae belong to Xanthophyta, lipid of dry microbial cells of *Schizochytrium* sp. belonging to Labyrinthulomycota, lipid of *Crypthecodinium cohnii,* butter lipid, ghee butter, milk fat, beef tallow, lard, sheep tallow, fats and oils of other land animals, fish oil, whale oil, codliver oil, fats and oils of other marine animals, short chain fatty acid-containing fats and oils, middle chain fatty acid-containing fats and oils, γ-oryzanol, γ-linolenic acid-containing fats and oils, α-linolenic acid-containing fats and oils, docosahexaenoic acid (DHA)-containing fats and oils, eicosapentaenoic acid (EPA)-containing fats and oils, plant sterol-containing fats and oils, trans acid-containing fats and oils, hydroxy acid-containing fats and oils, conjugated fatty acid-containing fats and oils, polyphenol-containing fats and oils, phospholipid-containing fats and oils, sphingolipids, tocotrienol-containing fats and oils, sitostanol fatty acid-containing fats and oils, n-3 polyunsaturated fatty acid-containing fats and oils, diglycerides, and other vegetable or animal fats and oils; processed oils derived from these fats and oils according to necessity, such as hydrogenated oils, slightly hydrogenated oils, hydrogenation isomerized oils, interesterified oils, and fractionated oils, fats and oils processed by two or more of these processes, and mixtures of two or more of these processed fats and oils. Additionally, emulsions (including W/O emulsions, O/W emulsions, double emulsions, i.e., O/W/O emulsions and W/O/W emulsions, and more complex multiple emulsions) of these edible fats and oils are also useful. Examples of the thickeners and stabilizers are pullulan, psyllium, gum arabic, gellan gum, glucomannan, guar gum, xanthan gum, tamarind gum, carrageenan, alginic acid salts, farceran, locust bean gum, pectin, curdlan, and low-molecular compounds obtained from these substances, starch, modified starch, gelatinized starch, crystalline cellulose, gelatin, dextrin, and agar. Additional useful food additives or foods include saccharides, such as glucose, maltose, enzyme-saccharified sugar (thick malt syrup), lactose, reducing starch saccharification products, isomerized liquid sugar, sucrose-coupled malt syrup, oligosaccharides, reducing sugar polydextrose, sorbitol, reduced lactose, trehalose, xylose, xylitol, maltitol, erythritol, mannitol, fructo-oligosaccharides, soybean oligosaccharides, galacto-oligosaccharides, lactosucrose-oligosaccharides, raffinose, lactulose, palatinose-oligosaccharides, stevia, and Aspartame; stabilizers, such as phosphoric acid salts (e.g., hexametaphosphorates, secondary phosphates and primary phosphates) and alkali metal (e.g., potassium or sodium) salts of citric acid; proteins, such as whey proteins, (e.g., α-lactalbumin, β-lactoglobulin, and serum albumin), casein and other milk proteins, low-density lipoprotein, high-density lipoprotein, egg proteins (e.g., phosvitin, livetin, phosphoglycoprotein, ovalbumin, conalbumin, and ovomucoid), wheat proteins (e.g., gliadin, glutenin, prolamine, and glutelin), and other vegetable and animal proteins; inorganic salts, such as sodium chloride, rock salt, sea salt, and potassium chloride; souring agents, such as acetic acid, lactic acid, and gluconic acid; colorants, such as β-carotin, caramel, and Monascus color; antioxidants, such as tocopherol and tea extract; eggs, such as whole eggs, egg yolk, egg white, and enzyme-processed eggs; cereals, such as bread flour, allpurpose flour, and cake flour; beans, such as soybean powder; water, flavors, dairy products, seasonings, pH adjustors, food preservatives, shelf life extenders, fruits, fruit juices, coffee, nut pastes, spices, cacao mass, and cocoa powder. Two or more of these additives can be used in combination or in the form of a complex thereof.

[0092] In addition to the above-mentioned materials, any other material that is usually used in bakery products and bakery product dough can be used in the bakery products and bakery product dough according to the present invention. The present invention is applicable to any breadmaking processes, such as a sponge-dough process and a straight

process. The time of adding the fat and oil composition of the present invention is not limited. Where the sponge-dough process is applied, the fat and oil composition may be added in the stage of preparing a sponge or in the stage of preparing a dough.

[0093] In the present invention it is preferred that the starch in the bakery product dough be partially gelatinized into α-starch before baking to enhance a favorable flavor or texture. The partial gelatinization can be effected by adding previously gelatinized starch of wheat, rice, maize, potato, cassava, taro, etc. or a raw material containing it to the dough or by adding starch or a raw material containing starch to the dough and gelatinizing the starch by heating, addition of hot water, steaming or roasting. For example, hot water can be added to part of wheat flour to convert it into α-starch, or part of wheat flour may be steamed.

[0094] Where applied to non-yeast bakery products, such as biscuits and pizza crusts, the fat and oil composition of the present invention gives an increased crispy texture.

[0095] The fat and oil composition applied to bakery products supplies β-glucans having excellent bioregulatory functions without being accompanied with reductions of taste and texture. Further, since the fat and oil composition produces the same effects as have been obtained by the conventional additives having various quality improving effects, it makes it possible to reduce the amounts of the conventional additives to be used, making contribution to improvement in taste and texture of bakery products without impairing the flavor of the products. Furthermore, the fat and oil composition makes it feasible to provide bakery products which meet the consumers's trends to naturally occurring substances, safety, and health.

[0096] Application of the fat and oil composition to confectionery products will then be described. In an embodiment of confectionery product production, dough is prepared by using the fat and oil composition containing β-glucans extracted from gramineous plants of the present invention as a part of or the whole of conventionally employed fats and oils, and the resulting dough is further processed. Confectionery products according to this embodiment include deep-fried products such as snacks and doughnuts and steamed products, such as steamed cakes and bean-jam buns. Confectionery products in another embodiment include candies, gums, chocolates, and tablets prepared by mixing the oil and fat composition containing β-glucans extracted from a gramineous plant with sugar, flavors, and the like and, if necessary, solidifying and molding the mixture. Cold desserts, such as ice cream and sherbet, are also included under confectionery products.

[0097] In making confectionery products, where weight is put on not only flavor but taste, particularly sweetness, it is important to eliminate lumps. Even a very small lump would cause a strange feeling and ruin the commercial value. Since the fat and oil composition according to the present invention previously contains the β-glucans extracted from a gramineous plant uniformly, even when it is added to and mixed with a premixed material, there is provided a final confectionery product which contains the β-glucans extracted from a gramineous plant in a uniformly dispersed state with no lumps, gives no strange feeling, and has a good flavor.

[0098] The fat and oil composition according to the present invention can be added to foods or drugs containing a food ingredient having a prophylactic action for habitual diseases (or life style-related diseases) to enhance the action. Example of such foods or drugs are those containing unsaturated higher fatty acids regulating a blood lipid concentration (e.g., EPA and DHA), plant sterols regulating blood serum cholesterol and esters thereof, diacylglycerol, γ-linolenic acid, α-linolenic acid, beet fiber, corn (maize) fiber, psyllium seed coat, tea polyphenol, lecithin; dried bonito peptide, sardine peptide, casein dodecapeptide, and soybean protein isolate which are effective in lowering blood pressure; and lactic acid bacteria, gluconic acid, oligosaccharides, and various dietary fibers which improve the intestinal environment to regulate the intestines. Further, foods or drugs with enhanced bioregulatory functions can be obtained by adding to the fat and oil composition of the present invention substances known to have health improving functionality, such as Chlorella, spirulina, propolis, chitin, chitosan, nucleic acids, leyss (*Ganoderma lucidum*), agaricus, ginkgo leaf extract, lakanka (Lo Hon Go), turmeric, garcinia, apple fiber, gymnema, collagen, blueberry, aloe, saw palmetto, plant fermentation enzyme, soybean isoflavon, chlorophyll, royal jelly, Asian ginseng, prune, and herbs, such as chamomile, thyme, sage, peppermint, lemon balm, mallow, oregano, cat nip tea, yarrow, and hibiscus.

[0099] When added to processed foods of rice, wheat, maize or soybeans, the fat and oil composition of the present invention is capable of imparting or enhancing functionality of these foodstuffs. Examples of such processed foods are rice products (e.g., boiled rice, rice cake, retort pouch boiled rice, frozen boiled rice, and sterile boiled rice); processed rice products, such as rice noodles, rice chips, and rice crackers; the above-recited bakery products and confectionery products; noodles, such as pastas, buckwheat noodles, *udon* noodles, *houtou* noodles, Chinese noodles, packaged instant noodles, and instant cup noodles; other wheat processed foods; breakfast cereals or processed maize products such as corn flakes; and processed soybean products, such as *tofu,* soybean milk, soybean milk beverages, *yuba* (soybean milk skin), thin fried *tofu,* thick fried *tofu,* round fried *tofu,* soybean jam, and *miso* (soybean paste). Additionally, the fat and oil composition can be added to a variety of foods, including dairy products, such as milk, processed milk, yogurt, whey beverages, fermented lactic acid beverages, butter, and cheese; Japanese sweets, such as *yokan* (bean jelly), *uiro* (steamed rice jelly), *monaka* (wafer with bean jam), and sweet bean jam; soups, such as potage, stew, and curry; seasonings, such as soy sauce, Worcester sauce, dips, jams, and tomato ketchup; processed meat products,

such as ham, sausage, and hamburgers; and processed marine products, such as steamed fish paste, baked fish paste, fried fish paste, and fish sausage.

[0100] Application of the fat and oil composition to liquid foods will then be described in detail.

[0101] The term "liquid foods" as used herein means not only liquids, such as soups and juices, but dried "liquid foods" in the form of ready-to-drink powder or solid that can be mixed with water or hot water into liquid, such as powdered soups and powdered juices.

[0102] The liquid food of the present invention can be obtained by adding the β-glucan-containing fat and oil composition to a liquid food, such as a beverage or soup. In adding, the mixture is mixed by stirring, if necessary, under heating. A conventional food emulsifier may be added to prepare an emulsion. The mixing means to be used is not particularly limited, and mixing devices, such as a mixer, can be used.

[0103] While the amount of the fat and oil composition to be added to a liquid food is not particularly limited, it is preferable to add the fat and oil composition in such an amount that the resulting liquid food may contain 0.1 to 30% by weight, particularly 0.5 to 20% by weight, of the extracted β-glucans. With an extracted β-glucan content less than 0.1% by weight, the β-glucans may fail to exhibit their functional effects. If the content exceeds 30% by weight, the extracted β-glucans can reduce the quality of the food as a whole.

[0104] In order to further suppress non-uniform distribution of the β-glucans or the liquid food containing the β-glucans due to lump formation, and the like, it is possible to add food additives or foods, such as emulsifiers, gelling agents, thickeners, and stabilizers, to the liquid food. These food additives or foods are not particularly limited as long as they are edible. Examples of the emulsifiers are lecithin, fatty acid monoglycerides, sorbitan fatty acid esters, propylene glycol fatty acid esters, sugar esters, polyoxyethylene sorbitan fatty acid esters, Tween, polyoxyethylene sorbitan trioleate, polyoxyethylene sorbitan monooleate, polyoxyethylene sorbitan monostearate, polyoxyethylene sorbitan monopalminate, and polyoxyethylene sorbitan monolaurate. Examples of the thickeners and stabilizers are pullulan, psyllium, gum arabic, gellan gum, glucomannan, guar gum, xanthan gum, tamarind gum, carrageenan, alginic acid salts, farceran, locust bean gum, pectin, curdlan, and low-molecular compounds obtained from these substances, starch, modified starch, gelatinized starch, crystalline cellulose, gelatin, dextrin, agar, and dextran. Additional useful food additives or foods include saccharides, such as glucose, fructose, sucrose, maltose, enzyme-saccharified sugar (thick malt syrup), lactose, reducing starch saccharification products, isomerized liquid sugar, sucrose-coupled malt syrup, oligosaccharides, reducing sugar polydextrose, sorbitol, reduced lactose, trehalose, xylose, xylitol, maltitol, erythritol, mannitol, fructo-oligosaccharides, soybean oligosaccharides, galacto-oligosaccharides, lactosucrose-oligosaccharides, raffinose, lactulose, palatinose-oligosaccharides, stevia, and Aspartame; stabilizers, such as phosphoric acid salts (e.g., hexametaphosphorates, secondary phosphates and primary phosphates) and alkali metal (e.g., potassium or sodium) salts of citric acid; proteins, such as whey proteins, (e.g., α-lactalbumin, β-lactoglobulin, and serum albumin), casein and other milk proteins, low-density lipoprotein, high-density lipoprotein, egg proteins (e.g., phosvitin, livetin, phosphoglycoprotein, ovalbumin, conalbumin, and ovomucoid), wheat proteins (e.g., gliadin, glutenin, prolamine, and glutelin), and other vegetable and animal proteins; protein-polysaccharide complexes, inorganic salts, such as sodium chloride, rock salt, sea salt, and potassium chloride, souring agents, such as acetic acid, lactic acid, and gluconic acid, colorants, such as β-carotin, caramel, and Monascus color, antioxidants, such as tocopherol and tea extract, eggs, such as whole eggs, egg yolk, egg white, and enzyme-processed eggs, cereals, such as bread flour, allpurpose flour, and cake flour, beans, such as soybean powder, water, flavors, dairy products, seasonings, pH adjustors, enzymes, food preservatives, shelf life extenders, fruits, fruit juices, coffee, nut pastes, spices, cacao mass, and cocoa powder. Two or more of these additives can be used in combination or in the form of a complex thereof. The amounts of the additives to be added are not particularly limited. They can be added in general amounts, for example, 0.01 to 15% by weight based on the resulting liquid food.

[0105] Examples of the liquid foods include soups, such as consomme, corn potage, egg soup, Chinese soups, stew, and curry, juices or fruit juice beverages, such as orange juice, tomato juice, banana juice, vegetable juice, fruit juices with fruit, and beverages containing fruit juice, carbonated beverages, such as cola and soda pop, milk beverages, such as milk, processed milk, yogurt, and whey drinks, seasonings, such as soy sauce, Worcester sauce, dips, jams, and tomato ketchup, coffee, cocoa, tea, green tea, woolong tea, soybean milk, fermented lactic acid beverages, alcoholic beverages, such as *sake*, whisky, brandy, beer, *sho-chu*, wines, and sparkling alcohol, vitamin rich drinks, health drinks, tonic drinks, jelly drinks, nutritious drinks, sports drinks, and coffee drinks. Also included are the above-mentioned dried liquid foods and ready-to-drink dried foods such as powdered soups which are mixed with hot water or water, etc. into liquid.

[0106] Application of the fat and oil composition to processed foods mainly comprising starch will then be described in detail.

[0107] The fat and oil composition is preferably used in an amount of 0.05 to 30% by weight, in terms of the extracted β-glucans, based on a processed food mainly comprising starch.

[0108] The processed food mainly comprising starch includes any food containing, as an ingredient, starch originated in rice, wheat, maize, potato, cassava, taro, and so forth. Examples of the processed foods include noodles, such as *udon* noodles, buckwheat noodles, Chinese noodles, chow mein noodles, sauteed *udon* noodles, spaghetti, pasta, raw

noodles, dried noodles, packaged instant noodles, and instant cup noodles; cooked rices, such as doria, pilaf, beef bowl, *tempura* bowl, chicken-and-egg bowl, curry and rice, boiled rice, sterile boiled rice, and retort pouch boiled rice; processed rice products, such as rice crackers, dumplings, rice chips, and rice cakes; fried foods, such as *korokke* (Japanese style croquettes), deep-fried port cutlets, and *tempura*, and their batters; steamed buns stuffed with sweet bean jam, pork, etc.; Chinese dishes, such as shui mai, pot-stickers, spring rolls, and other Chinese snacks; Japanese pancakes, such as *okonomi-yaki* or *monja-yaki* (As-you-like-it pancakes), and octopus pancakes; and premixes for pizza, pancakes, doughnuts, *tempura*, etc. Application to the aforementioned processed foods, such as frozen foods, retorted foods, and microwave foods, is also preferred. As a matter of course, application to foods which are to be heated or thawed in a microwave oven is also preferred.

[0109] The present invention will now be illustrated in greater detail with reference to Examples, but it should be understood that the present invention is by no means limited thereto. Unless otherwise noted, all the parts and percents are by weight.

Test Example 1: Measurement of β-glucan content

[0110] Analysis of β-glucans was carried out according to the McCleary method (enzyme assay) with a β-glucan assay kit from MegaZyme. Where a sample under analysis is powder, it was sieved through a 500 μm (30 mesh) sieve, and the water content was measured. A 100 mg portion of the sample was put into each 17 ml test tube, and 200 μl of a 50% ethanol solution was added thereto to disperse the powder. Then 4 ml of a 20 mM phosphate buffer (pH 6.5) was added, and the mixture was mixed well. The mixture was heated in a boiling water bath for 1 minute. The mixture was mixed thoroughly and further heated in a hot water bath for 2 minutes. After the mixture was cooled to 50°C and allowed to stand for 5 minutes, 200 μl (10 U) of a lichenase solution was added to each tube. The lichenase solution was prepared by diluting the lichenase vial attached to the kit with 20 ml of the 20 mM phosphate buffer, and the rest of the enzyme solution was preserved in a freezer. The mixture was allowed to react at 50°C for 1 hour. Then, 5 ml of a 200 mM acetate buffer (pH 4.0) was added to each tube, followed by mixing gently. After allowing the tubes at room temperature for 5 minutes, the system was centrifuged to obtain the supernatant liquor. The supernatant liquor (100 μl) was taken into three test tubes. To one of them, 100 μl of a 50 mM acetate buffer (pH 4.0) was added, and to each of the other two was added 100 μl (0.2 U) of a β-glucosidase solution, which was prepared by diluting the glucosidase vial attached to the kit with 20 ml of the 50 mM acetate buffer. The rest of the enzyme solution was kept in a freezer. The system was allowed to react at 50°C for 10 minutes. A glucose oxidase/peroxidase solution (3 ml) was added, followed by reacting at 50°C for 20 minutes. The absorbance (EA) of each sample at 510 nm was measured. The β-glucan content was obtained from equation:

$$\beta\text{-Glucan content (w/w \%)} = (EA) \times (F/W) \times 8.46$$

where F: 100/absorbance of 100 μg glucose
W: calculated weight (mg) of anhydride

[0111] Where a sample under analysis was a liquid extract containing extracted β-glucans, the liquid was made into solid or powder as follows before analysis. To a β-glucan extract was added twice as much ethanol, and the mixture was stirred well and centrifuged. The precipitate was collected, dried well, and ground to prepare a solid β-glucan extract. After the water content of the β-glucan extract was measured, the sample was analyzed by the McCleary method (enzyme assay) using a β-glucan assay kit from MegaZyme. Each precipitate sample weighing 50 mg was put into a 17 ml test tube and dispersed in 200 μl of a 50% ethanol solution. The dispersion was analyzed in the same manner as described above.

Test Example 2: Measurement of molecular weight

[0112] The molecular weight of an extract was measured as follows. A solid extract weighing 5 mg was put into a test tube, 0.5 ml of distilled water was added thereto and heated to dissolve the extract in a boiling water bath. The solution was filtered through a 0.22 μm filter to prepare a sample for HPLC. The sample was analyzed by HPLC using a gel-filtration column, Shodex-packed column KS-805 (available from Showa Denko K.K.) at a flow rate of 0.6 ml/min at a temperature of 50°C. An RI detector was used for detection, water was used as a developing solvent, and Shodex Pullulan Standards P-82 (available from Showa Denko K.K.) was used as a molecular weight marker.

[0113] Where extracted β-glucans were a liquid extract, it was diluted with twice as much ethanol, cooled to -20°C, and allowed to stand for 1 hour to obtain a precipitate. A 5 mg portion of the precipitate was put into a tube, which was analyzed in the same manner as described above to measure the molecular weight.

Preparation Example 1: Preparation of raw material and extraction accelerator

[0114] Glutinous naked barley was polished to a polishing yield of 82%. The bran generated by this first polishing was designated bran-1. The barley having been polished to a polishing degree of 82% was further polished in a grinding type polishing machine to a polishing yield of 55%. The bran generated by the second polishing was designated grinds-1. In a 50 liter container was put 20 liters tap water and adjusted to 15°C while stirring. Six kilograms of bran-1 was added thereto and extracted while stirring for 2 hours. The mixture was centrifuged in a continuous centrifuge for solid-liquid separation, and the supernatant liquor was lyophilized to obtain 450 g of an extraction accelerator.

Preparation Example 2: Preparation of β-glucans

[0115] Into a 70 liter container was put 30 liters tap water, and 150 g of the extraction accelerator prepared in Preparation Example 1 was added. After dissolution, 7.5 kg of grinds-1 was added, followed by extraction by stirring at 50°C for 2 hours. The mixture was centrifuged for solid-liquid separation in a continuous centrifuge. The supernatant liquor separated was boiled and cooled to give 15 liters of a slightly viscous β-glucan solution (sample 1). As a result of analysis according to Test Example 1, the β-glucan content was found to be 3%. As a result of analysis according to Test Example 2, the extract was detected in the molecular weight range of from 10,000 to 90,000, with the maximum peak at 40,000. The maximum peak was confirmed to be β-glucans by the method of Test Example 1.

Preparation Example 3: Preparation of β-glucans

[0116] To a β-glucan solution prepared in the same manner as in Preparation Example 2 was added twice as much ethanol. A precipitate thus formed was collected and dried to give 460 g of a β-glucan extract (sample 2), which was found to have a β-glucan purity of 91% as a result of analysis according to Test Example 1. As a result of analysis according to Test Example 2, the extract was detected in the molecular weight range of from 10,000 to 200,000, with the maximum peak at 40,000. The maximum peak was confirmed to be β-glucans by the method of Test Example 1.

Preparation Example 4: Preparation of β-glucans

[0117] A β-glucan solution prepared in the same manner as in Preparation Example 2 was lyophilized as such to give a β-glucan extract (sample 3) weighing 580 g. Sample 3 was found to have a β-glucan purity of 76% as a result of analysis according to Test Example 1. As a result of analysis according to Test Example 2, the extract was detected in the molecular weight range of from 10,000 to 200,000, with the maximum peak at 40,000. The maximum peak was confirmed to be β-glucans by the method of Test Example 1.

Preparation Example 5: Preparation of β-glucans

[0118] Into a 70 liter container was put 30 liters tap water, and 60 g of sodium hydroxide was added thereto while stirring. After dissolution, 7 kg of grinds-1 was added, followed by extraction by stirring at 30°C for 2 hours. The mixture was neutralized with hydrochloric acid and separated into solid and liquid by means of a continuous centrifuge. The supernatant liquor separated was boiled to give 15 liters of a slightly viscous β-glucan solution (sample 4). As a result of analysis according to Test Example 1, the β-glucan content was found to be 1.8 wt%. As a result of analysis according to Test Example 2, the extract showed no peak in a molecular weight range between 3000 and 100,000 but an extremely broad peak in the range of from 100,000 to 500,000. The fraction collected in the molecular weight range of 100,000 or greater was confirmed to be β-glucans by the method of Test Example 1.

Method of evaluation in Examples 1 to 32 and Comparative Examples 1 to 23:

[0119] In Examples 1 to 32 and Comparative Examples 1 to 23 hereinafter described, evaluation was made in terms of stability and texture (smoothness, hardness, and flavor) according to necessity. Stability was evaluated by visually inspecting any change in appearance after storage at 5°C for 1 month. Texture was organoleptically evaluated by 10 panel members and rated A to C according to standards shown below. The rating given to a sample by the greatest number out of 10 panel members was the rating of the sample. The results obtained are shown in Tables 1 and 2, in which marks "-" indicate no evaluation made.

Standards of evaluation:

[0120]

1) Stability

A: Excellent in stability.
B: Change in appearance, such as slight phase separation, observed.
C: Phase separation observed.

2) Texture
2-1) Smoothness

A: Very smooth
B: Smooth
C: Not smooth

2-2) Hardness

A: Very soft
B: Soft
C: Not soft

2-3) Flavor

A: Superior
B: Slightly inferior
C: Inferior

Example 1: Fat and oil composition containing β-glucans extracted from gramineous plant

[0121] A hundred parts of sample 2 obtained in Preparation Example 3 and 100 parts of soybean oil were thoroughly mixed in a kneader. The mixture was kept at 60°C for 10 minutes and then cooled to room temperature, whereupon it became creamy to give a fat and oil composition-1 containing β-glucans extracted from a gramineous plant according to the present invention (β-glucan content: 45.50%). The β-glucans were found uniformly dispersed.

Example 2: Fat and oil composition containing β-glucans extracted from gramineous plant

[0122] Three hundred parts of sample 3 obtained in Preparation Example 4 was mixed with 100 parts of palm oil melted by heating at 70°C and 1 part of lecithin in a high-speed homomixer. The mixture was left to stand at 50°C for 20 minutes and cooled to room temperature to give a lumpy fat and oil composition-2 containing β-glucans extracted from a gramineous plant according to the present invention (β-glucan content: 56.90%). The β-glucans were found uniformly dispersed.

Example 3: Fat and oil composition containing β-glucans extracted from gramineous plant

[0123] Fifty parts of sample 3 obtained in Preparation Example 4 was mixed with 30 parts of palm olein oil, 70 parts of rapeseed oil, and 0.2 part of protease-hydrolyzed egg yolk in a mixer. The mixture was left to stand at 65°C for 15 minutes and cooled to room temperature to give a creamy fat and oil composition-3 containing β-glucans extracted from a gramineous plant according to the present invention (β-glucan content: 25.30%). The β-glucans were found uniformly dispersed.

Example 4: Fat and oil composition containing β-glucans extracted from gramineous plant

[0124] Five parts of sample 2 obtained in Preparation Example 3 was mixed with 40 parts of rice oil, 20 parts of olive oil, and 35 parts of safflower oil in a high-speed homomixer. The mixture was allowed to stand at 50°C for 30 minutes and cooled to room temperature to give a fat and oil composition-4 containing β-glucans extracted from a gramineous plant according to the present invention (β-glucan content: 4.60%), which had almost the same viscosity as the starting oils but showed slight turbidity. The β-glucans were found uniformly dispersed.

Example 5: Fat and oil composition containing β-glucans extracted from gramineous plant

[0125] Thirteen parts of sample 1 obtained in Preparation Example 2 was mixed with 20 parts of hydrogenated soybean oil (melting point: 45°C), 35 parts of palm oil, 30 parts of cotton seed oil, and 0.2 part of soybean lysolecithin. The mixture was allowed to stand at 70°C for 10 minutes and then emulsified in a high-speed mixer, followed by rapidly cooling for plasticization to give a fat and oil composition-5 containing β-glucans extracted from a gramineous plant according to the present invention (β-glucan content: 0.40%), which had margarine-like physical properties. The β-glucans were found uniformly dispersed.

Example 6: Fat and oil composition containing β-glucans extracted from gramineous plant

[0126] Fifty parts of sample 4 obtained in Preparation Example 5 was mixed with 27.6 parts of hydrogenated fish oil (melting point: 36°C), 18 parts of corn salad oil, and 0.4 part of glycerol monotartrate by stirring at 50°C for 30 minutes. The mixture was emulsified in a high-speed mixer, followed by rapidly cooling for plasticization to give a fat and oil composition-6 containing β-glucans extracted from a gramineous plant according to the present invention (β-glucan content: 0.94%), which had fat spread-like physical properties. The β-glucans were found uniformly dispersed.

Example 7: Fat and oil composition containing β-glucans extracted from gramineous plant

[0127] Twenty parts of sample 1 obtained in Preparation Example 2 was mixed with 0.3 part of olive oil (melting point: 36°C) and 0.1 part of casein sodium. The mixture was allowed to stand at 55°C for 15 minutes, emulsified in a high-speed mixer, and spray-dried to give a powdered fat and oil composition-7 containing β-glucans extracted from a gramineous plant according to the present invention (β-glucan content: 60.00%). The β-glucans were found uniformly dispersed.

Example 8: Production of shortening

[0128] An oil phase consisting of 30 parts of palm oil, 50 parts of hydrogenated palm oil, 20 parts of rapeseed oil, and 0.3 part of lecithin was melted at 70°C. To 100 parts of the oil phase was added 5.0 parts of sample 2 obtained in Preparation Example 3, and the mixture was allowed to stand at 70°C for 30 minutes. The mixture was then stirred in a homomixer at a high rotational speed for 2 minutes to give a fat and oil composition-8 containing β-glucans extracted from a gramineous plant according to the present invention. The β-glucans were found by visual observation sufficiently dispersed in the fat and oil. The composition was rapidly cooled for plasticization and cooled to 5°C to obtain shortening (β-glucan content: 4.30%) according to the present invention. The shortening of the present invention was evaluated for smoothness and flavor. The results obtained are shown in Table 1. It is seen that the resulting shortening is superior to Comparative Example 1 described hereunder in smoothness and flavor. Although the step of crystal aging had been omitted, the shortening of the present invention can be said to have enjoyed the effects of forming moderate crystals with excellent texture, accelerating such crystallization, and preventing reduction of flavor that might have been caused by an emulsifier.

Comparative Example 1: Production of shortening

[0129] An oil phase consisting of 30 parts of palm oil, 50 parts of hydrogenated palm oil, 20 parts of rapeseed oil, and 0.3 part of lecithin was melted at 70°C, stirred in a homomixer at a high rotational speed for 2 minutes, rapidly cooled for plasticization, and cooled to 5°C to obtain an edible fat and oil composition. The resulting shortening was evaluated for smoothness and flavor for comparison. The results obtained are shown in Table 2, which prove the resulting shortening to be much inferior in flavor.

Example 9: Production of margarine

[0130] A hundred part of an edible fat and oil consisting of palm oil, hydrogenated palm oil, rapeseed oil, and sorbitan fatty acid esters at a weight ratio of 30:50:20:0.3 was melted at 70°C. Eight parts of sample 3 obtained in Preparation Example 4 was added thereto, and the mixture was left to stand at 65°C for 30 minutes. A solution of 0.5 part of defatted milk powder and 1 part of edible salt in 16 parts of hot water (70°C) was slowly added to the mixture and mixed therewith while stirring in a homomixer. Then, the mixture was rapidly cooled to plasticize, maintained at 25°C overnight, and cooled to 5°C to give margarine according to the present invention (β-glucan content: 4.80%). The β-glucans were found uniformly dispersed. The margarine of the present invention was evaluated for stability, smoothness, and flavor. The results are shown in Table 1. The resulting margarine had a fine and smooth texture. Further, the margarine was more flavorful than that of Comparative Example 2 described hereunder, proving the effect of suppressing a reduction in flavor

which might have been caused by the emulsifier.

Comparative Example 2: Production of margarine

[0131] A hundred part of an edible fat and oil consisting of palm oil, hydrogenated palm oil, rapeseed oil, and sorbitan fatty acid esters at a weight ratio of 30:50:20:0.3 was melted at 70°C. To the mixture was slowly added a solution of 0.5 part of defatted milk powder and 1 part of edible salt in 16 parts of hot water (70°C) while stirring in a homomixer. After mixing, the mixture was rapidly cooled to plasticize, maintained at 25°C overnight, and cooled to 5°C to give margarine. The resulting margarine was evaluated for stability, smoothness, and flavor for comparison. The results are shown in Table 2.

Example 10: Production of dressing

[0132] Ten parts of sample 2 prepared in Preparation Example 3, 10 parts of egg yolk, 1.5 parts of edible salt, 11 parts of vinegar, 2.5 parts of soft white sugar, 0.05 part of mustard powder, and 0.05 part of onion powder were mixed by stirring in a mixer at a high speed for 5 minutes to prepare an aqueous phase. While the aqueous phase was further stirred in a homomixer at a high speed, 75 parts of soybean salad oil heated to 70°C was slowly added thereto and mixed. The mixture was left to stand at 50°C for 10 minutes, emulsified, and cooled at 5°C for 24 hours to obtain a dressing (β-glucan content: 8.27%) according to the present invention. The β-glucans were found uniformly dispersed. The dressing of the present invention was evaluated for stability and flavor. The results are shown in Table 1. The resulting dressing was proved excellent in stability and flavor.

Comparative Example 3: Production of dressing

[0133] Ten parts of egg yolk, 1.5 parts of edible salt, 11 parts of vinegar, 2.5 parts of soft white sugar, 0.05 part of mustard powder, and 0.05 part of onion powder were mixed by stirring in a mixer at a high speed for 5 minutes to prepare an aqueous phase. A dressing was prepared by using the aqueous phase in the same manner as in Example 10. The resulting dressing was evaluated for stability and flavor for comparison. The results are shown in Table 2.

Example 11: Production of dressing

[0134] Ten parts of egg yolk, 1.5 parts of edible salt, 11 parts of vinegar, 2.5 parts of soft white sugar, 0.05 part of mustard powder, and 0.05 part of onion powder were mixed by stirring in a mixer at a high speed for 5 minutes to prepare an aqueous phase. While the aqueous phase was further stirred in a homomixer at a high speed, 75 parts of the fat and oil composition-4 containing β-glucans extracted from a gramineous plant which was prepared in Example 4 was slowly added thereto. The mixture was emulsified and cooled at 5°C for 24 hours to give a dressing (β-glucan content: 3.45%) according to the present invention. The β-glucans were found uniformly dispersed. The dressing of the present invention was evaluated for stability and flavor. The results are shown in Table 1. The resulting dressing was proved excellent in stability and flavor.

Comparative Example 4: Production of dressing

[0135] Ten parts of egg yolk, 1.5 parts of edible salt, 11 parts of vinegar, 2.5 parts of soft white sugar, 0.05 part of mustard powder, and 0.05 part of onion powder were mixed by stirring in a mixer at a high speed for 5 minutes to prepare an aqueous phase. While the aqueous phase was further stirred in a homomixer at a high speed, 75 parts of a fat and oil mixture consisting of 40 parts of rice oil, 20 parts of olive oil, and 35 parts of safflower oil was slowly added thereto. The mixture was emulsified and cooled at 5°C for 24 hours to give a dressing, which was evaluated for stability and flavor for comparison. The results are shown in Table 2.

Example 12: Production of mayonnaise

[0136] Thirty parts of soybean salad oil was added to 30 parts of sample 1 obtained in Preparation Example 2, and the mixture was stirred for preliminary emulsification to prepare a fat and oil composition containing β-glucans extracted from a gramineous plant according to the present invention. A thoroughly stirred mixture consisting of 9 parts of egg yolk, 5.2 parts of starch, 8.2 parts of sugar, 2.8 parts of edible salt, 8 parts of vinegar, 1 part of seasoning spices, and 6 parts of water was added to the fat and oil composition, and the mixture was finally emulsified in a colloid mill to give mayonnaise (β-glucan content: 0.09%) according to the present invention. The β-glucans were found uniformly dispersed. The mayonnaise of the present invention was evaluated for stability, smoothness, and flavor. The results obtained are

shown in Table 1. The resulting mayonnaise underwent no separation of water when stored for 1 month and had a smooth texture and a very good flavor.

Comparative Example 5: Production of mayonnaise

[0137] Thirty parts of soybean salad oil was added to 30 parts of water, and the mixture was stirred for preliminary emulsification to prepare a fat and oil composition. A thoroughly stirred mixture consisting of 9 parts of egg yolk, 5.2 parts of starch, 8.2 parts of sugar, 2.8 parts of edible salt, 8 parts of vinegar, 1 part of seasoning spices, and 6 parts of water was added to the fat and oil composition, and the mixture was finally emulsified in a colloid mill to prepare mayonnaise, which was evaluated for stability, smoothness and flavor for comparison. The results obtained are shown in Table 2.

Example 13: Production of mayonnaise

[0138] Nine parts of egg yolk, 8.2 parts of sugar, 2.8 parts of edible salt, 8 parts of vinegar, 1 part of seasoning spices, and 36 parts of sample 4 obtained in Preparation Example 5 were mixed to prepare an aqueous phase. To the aqueous phase were added 25 parts of rapeseed oil and 10 parts of the fat and oil composition of Example 1, and the mixture was preliminarily emulsified by stirring and finally emulsified in a colloid mill to prepare mayonnaise (β-glucan content: 5.20%) according to the present invention. The β-glucans were found uniformly dispersed. The mayonnaise of the present invention was evaluated for stability, smoothness, and flavor. The results obtained are shown in Table 1. The resulting mayonnaise underwent no separation of water when stored for 1 month and had a smooth texture and a very good flavor.

Comparative Example 6: Production of mayonnaise

[0139] Nine parts of egg yolk, 8.2 parts of sugar, 2.8 parts of edible salt, 8 parts of vinegar, 1 part of seasoning spices, and 36 parts of water were mixed to prepare an aqueous phase. To the aqueous phase were added 25 parts of rapeseed oil and 10 parts of palm oil, and the mixture was preliminarily emulsified by stirring and finally emulsified in a colloid mill to prepare mayonnaise, which was evaluated for stability, smoothness, and flavor for comparison. The results obtained are shown in Table 2.

Example 14: Production of fat spread

[0140] A mixture consisting of 27.6 parts of hydrogenated fish oil (melting point: 36°C), 18.4 parts of cotton seed oil, 40 parts of sample 1 obtained in Preparation Example 2, 12.3 parts of water, 1 part of edible salt, 0.5 part of defatted milk powder, 0.2 part of flavors, and 0.3 part of lecithin was emulsified and rapidly cooled for plasticization to prepare fat spread (β-glucan content: 1.20%) according to the present invention. The β-glucans were found uniformly dispersed. The fat spread of the present invention was evaluated for stability, smoothness, and flavor. The results are shown in Table 1. The resulting fat spread underwent no separation of water when stored for 1 month and had a smooth texture and a very good flavor.

Comparative Example 7: Preparation of fat spread

[0141] A mixture consisting of 27.6 parts of hydrogenated fish oil (melting point: 36°C), 18.4 parts of cotton seed oil, 52.3 parts of water, 1 part of edible salt, 0.5 part of defatted milk powder, 0.2 part of flavors, and 0.3 part of lecithin was emulsified and rapidly cooled for plasticization to prepare fat spread, which was evaluated for stability, smoothness and flavor for comparison. The results are shown in Table 2.

Example 15: Production of curry sauce mix

[0142] Forty-four parts of wheat flour (cake flour) and 34 parts of the shortening obtained in Example 8 were pan-fried brown, and the resulting roux was mixed with 8 parts of a commercially available curry powder mix to obtain a curry sauce mix (β-glucan content: 1.70%) according to the present invention. The β-glucans were found uniformly dispersed.

Example 16: Production of cookies

[0143] Fifty parts of the fat and oil composition-3 containing β-glucans extracted from a gramineous plant obtained in Example 3 and 50 parts of soft white sugar were kneaded into cream in a hobart mixer at a high speed for 6 minutes.

A mixture of 15 parts net of whole eggs, 1 part of edible salt, and 0.5 part of ammonium hydrogencarbonate was added thereto, followed by mixing at a medium speed for 30 seconds. A hundred parts of sieved wheat flour was added, followed by mixing at a low speed for 30 seconds to prepare dough. The dough was put into a cylinder of 6 cm in diameter, pressed out by 1 cm, and cut. The cut pieces of the dough were baked at 200°C for 13 minutes to obtain cookies (β-glucan content: 5.86%) according to the present invention. The β-glucans were found uniformly dispersed. The cookies of the present invention were evaluated for hardness and flavor. The results are shown in Table 1.

Comparative Example 8: Production of cookies

[0144] Fifty parts of a fat and oil mixture consisting of 30 parts of palm olein oil, 70 parts of rapeseed oil, and 0.2 part of protease-hydrolyzed egg yolk and 50 parts of soft white sugar were kneaded into cream in a hobart mixer at a high speed for 6 minutes. A mixture of 15 parts net of whole eggs, 1 part of edible salt, and 0.5 part of ammonium hydrogencarbonate was added thereto, followed by mixing at a medium speed for 30 seconds. The mixture was further processed in the same manner as in Example 16 to obtain cookies, which were evaluated for hardness and flavor for comparison. The results are shown in Table 2.

Example 17: Production of cookies

[0145] Fifty parts of the fat and oil composition-5 containing β-glucans extracted from a gramineous plant obtained in Example 5 and 40 parts of soft white sugar of beet were kneaded into cream in a hobart mixer at a high speed for 6 minutes. Twenty parts of raisin paste was added and mixed into the cream at a medium speed for 30 seconds. Sieved foxtail millet flour was added, followed by mixing at a low speed for 30 seconds to prepare dough. The dough was put into a cylinder of 6 cm in diameter, pressed out by 1 cm, and cut. The cut pieces of the dough were baked at 160°C for 15 minutes to obtain cookies (β-glucan content: 0.10%) according to the present invention. The β-glucans were found uniformly dispersed. The cookies of the present invention were evaluated for hardness and flavor. The results are shown in Table 1. The resulting cookies had a satisfactory texture despite the fact that neither eggs nor dairy products was used.

Comparative Example 9: Production of cookies

[0146] A mixture of 20 parts of hydrogenated soybean oil (melting point: 45°C), 35 parts of palm oil, 30 parts of cotton seed oil, and 0.2 part of soybean lysolecithin was allowed to stand at 70°C for 10 minutes and then emulsified in a high-speed mixer, followed by rapid cooling for plasticization to prepare an edible fat and oil composition having margarine-like physical properties. Fifty parts of the fat and oil composition and 40 parts of soft white sugar of beet were kneaded into cream in a hobart mixer at a high speed for 6 minutes. Twenty parts of raisin paste was added, followed by mixing at a medium speed for 30 seconds. The resulting mixture was further processed in the same manner as in Example 17 to obtain cookies, which were evaluated for hardness and flavor for comparison. The results are shown in Table 2.

Example 18: Production of chocolate

[0147] Twelve parts of cacao mass, 45 parts of powdered sugar, 20 parts of whole milk powder, 13 out of 23 parts of cacao butter, and 2 parts of sample 2 of Preparation Example 3 were put into a hobart mixer and mixed with a beater at a medium speed for 3 minutes. The mixture was rolled and conched to prepare a fat and oil composition containing β-glucans extracted from a gramineous plant according to the present invention. As observed with the naked eye, the β-glucans were found uniformly dispersed. The rest of cacao butter was added thereto and mixed to obtain chocolate mass. The chocolate mass was subjected to tempering, poured into a mold, and cooled down to obtain chocolate of the present invention, which was evaluated for smoothness, hardness, and flavor. The results obtained are shown in Table 1. The resulting chocolate had good melt in the mouth and a good flavor.

Comparative Example 10: Production of chocolate

[0148] Twelve parts of cacao mass, 45 parts of powdered sugar, 20 parts of whole milk powder, 13 out of 23 parts of cacao butter, and 2 parts of a fat and oil composition consisting of 30 parts of palm olein oil, 70 parts of rapeseed oil, and 0.2 part of protease-processed egg yolk were put into a hobart mixer and mixed with a beater at a medium speed for 3 minutes. The mixture was rolled and conched to prepare a fat and oil composition. The rest of cacao butter was added thereto and mixed to obtain chocolate mass. The chocolate mass was further processed in the same manner as in Example 18 to obtain chocolate, which was evaluated for smoothness, hardness, and flavor for comparison. The results obtained are shown in Table 2.

Example 19: Production of chocolate

[0149] Twelve parts of cacao mass, 45 parts of powdered sugar, 20 parts of whole milk powder, 23 parts of cacao butter, and 20 parts of the fat and oil composition-2 containing β-glucans extracted from a gramineous plant prepared in Example 2 were put into a hobart mixer and mixed with a beater at a medium speed for 3 minutes. The mixture was rolled and conched to obtain chocolate mass. The chocolate mass was subjected to tempering, poured into a mold, and cooled down to obtain solid chocolate of the present invention. The β-glucans were found uniformly dispersed. The chocolate of the present invention was evaluated for smoothness, hardness, and flavor. The results are shown in Table 1. The resulting chocolate had good melt in the mouth and a good flavor.

Comparative Example 11: Production of chocolate

[0150] Twelve parts of cacao mass, 45 parts of powdered sugar, 20 parts of whole milk powder, 23 parts of cacao butter, and 20 parts of palm oil were put into a hobart mixer. The same procedures of Example 19 were then followed to obtain a chocolate product, which was evaluated for smoothness, hardness, and flavor for comparison. The results obtained are shown in Table 2.

Example 20: Production of bread

[0151] Bread was made by using the β-glucan-containing margarine obtained in Example 9. A hundred parts of wheat flour, 3 parts of yeast, 4 parts of sugar, 2 parts of edible salt, 6 parts of the margarine obtained in Example 9, and 60 parts of water were mixed in a hopper mixer at a mixing temperature of 28°C at a low speed for 2 minutes and at a high speed for 4 minutes to prepare bread dough. The dough was allowed to ferment at 28°C for 60 minutes and divided into 450 g portions, which were each formed into a ball and allowed to prove at 28°C for 20 minutes. The dough was passed through a sheeter three times, shaped, put into a one-loaf pan, and finally proofed at 38°C and 90% RH until it rose 2 cm above the lip of the pan. The final proofing took 42 minutes. The proofed dough was baked at 220°C for 23 minutes to obtain a loaf of bread (β-glucan content: 2.10%) according to the present invention. The β-glucans were found uniformly dispersed. The bread of the present invention was evaluated for hardness and flavor. The results obtained are shown in Table 1. The resulting bread had softness, good volume and a satisfactory texture.

Comparative Example 12: Production of bread

[0152] Bread was made in the same manner as in Example 20, except for replacing the margarine used in Example 20 with margarine which was prepared in the same manner as in Example 9 except for using no β-glucans. The resulting bread was evaluated for hardness and flavor for comparison. The results obtained are shown in Table 2.

Example 21: Production of bread

[0153] A hundred parts of wheat flour, 3 parts of yeast, 4 parts of sugar, 2 parts of edible salt, 2 parts of the powdered fat and oil obtained in Example 7, 4 parts of shortening, 50 parts of the sample obtained in Preparation Example 2, and 13 parts of water were mixed in a hopper mixer at a mixing temperature of 28°C at a low speed for 2 minutes and at a high speed for 4 minutes to prepare bread dough. The dough was allowed to ferment at 28°C for 60 minutes and divided into 450 g portions, which were each formed into a ball and allowed to prove at 28°C for 20 minutes. The dough was passed through a sheeter three times, shaped, put into a one-loaf pan, and finally proofed at 38°C and 90% RH until it rose 2 cm above the lip of the pan. The final proofing took 46 minutes. The proofed dough was baked at 210°C for 30 minutes to obtain a loaf of bread (β-glucan content: 2.00%) according to the present invention. The β-glucans were found uniformly dispersed. The bread of the present invention was evaluated for hardness and flavor. The results obtained are shown in Table 1. The resulting bread had softness, good volume and a satisfactory texture.

Comparative Example 13: Production of bread

[0154] A hundred parts of wheat flour, 3 parts of yeast, 4 parts of sugar, 2 parts of edible salt, 2 parts of a powdered fat and oil prepared in the same manner as in Example 7 except for using no β-glucans, 4 parts of shortening, and 63 parts of water were mixed in a hopper mixer at a mixing temperature of 28°C at a low speed for 2 minutes and at a high speed for 4 minutes to prepare bread dough. The dough was further processed in the same manner as in Example 21 to obtain a loaf of bread, which was evaluated for hardness and flavor for comparison. The results are shown in Table 2.

Example 22: Making of boiled rice

[0155] Japonica rice cultivar Koshihikari made in Niigata was washed well with water. To 100 parts of washed rice were added 60 parts of water and 4 parts of the fat and oil composition-1 containing β-glucans extracted from a gramineous plant which was obtained in Example 1. The rice was boiled in an electric rice cooker to obtain boiled rice (β-glucan content: 1.10%). The β-glucans were found uniformly dispersed. The boiled rice was evaluated for hardness. The results are shown in Table 1. The resulting boiled rice had a light and soft texture.

Comparative Example 14: Making of boiled rice

[0156] Japonica rice cultivar Koshihikari made in Niigata was washed well with water. To 100 parts of washed rice were added 60 parts of water and 4 parts of soybean oil. The rice was boiled in an electric rice cooker to obtain boiled rice, which was evaluated for hardness for comparison. The results are shown in Table 2.

Example 23: Production of popcorn

[0157] Into a pan were put 100 parts of popcorn kernels, 2 parts of edible salt, and 10 parts of the fat and oil composition-4 containing β-glucans extracted from a gramineous plant obtained in Example 4, and the pot covered with a lid was heated on fire to obtain popcorn (β-glucan content: 0.41%). The β-glucans were found uniformly dispersed. The popcorn of the present invention was evaluated for texture. The results are shown in Table 1. The resulting popcorn had good quality with a smooth and light texture.

Example 24: Production of *tofu*

[0158] *Tofu* was produced by using the shortening prepared in Example 8. Soybeans were soaked in water. A hundred parts of soaked soybeans was ground together with 140 parts of water, boiled at 100°C for 5 minutes, put into a cotton bag, and squeezed to obtain soy milk. To the soy milk were added 3 parts of a coagulant (calcium sulfate) and 10 parts of the shortening obtained in Example 8. The mixture was gently stirred, coagulated at 75°C, and poured into a strainer lined with cotton cloth, left to stand for 30 minutes to obtain *tofu* (β-glucan content: 1.70%) according to the present invention. The β-glucans were found uniformly dispersed. The resulting *tofu* was evaluated for smoothness and flavor. The results are shown in Table 1. The resulting *tofu* had a good texture.

Comparative Example 15: Production of *tofu*

[0159] Soybeans were soaked in water. A hundred parts of soaked soybeans was ground together with 140 parts of water, boiled at 100°C for 5 minutes, put into a cotton bag, and squeezed to obtain soy milk. To the soy milk were added 3 parts of a coagulant (calcium sulfate) and 10 parts of shortening prepared in the same manner as in Example 8 except for using no β-glucans. The mixture was gently stirred, coagulated at 75°C, and poured into a strainer lined with cotton cloth, left to stand for 30 minutes to obtain *tofu*, which was evaluated for smoothness and flavor for comparison. The results are shown in Table 2.

Example 25: Production of soft chocolate

[0160] A mixture consisting of 50 parts of sugar, 5 parts of cacao mass, 15 parts of whole fat milk powder, 30 parts of the fat and oil composition-2 containing β-glucans extracted from a gramineous plant which was obtained in Example 2, 0.3 part of lecithin, and 0.04 part of vanillin was subjected to rolling and conching in a usual manner to obtain soft chocolate (β-glucan content: 17%) according to the present invention. The β-glucans were found uniformly dispersed. The soft chocolate of the present invention was evaluated for smoothness, hardness, and flavor. The results obtained are shown in Table 1. The resulting soft chocolate underwent no blooming and had a good flavor.

Comparative Example 16: Production of soft chocolate

[0161] A mixture consisting of 50 parts of sugar, 5 parts of cacao mass, 15 parts of whole fat milk powder, 30 parts of palm oil, 0.3 part of lecithin, and 0.04 part of vanillin was subjected to rolling and conching in a usual manner to obtain soft chocolate, which was evaluated for smoothness, hardness, and flavor for comparison. The results obtained are shown in Table 2.

Example 26: Production of water-free cream

[0162] Thirty-five parts of the fat and oil composition-8 containing β-glucans extracted from a gramineous plant which was obtained in Example 8, 45 parts of sugar, 10 parts of a tasty powder, and 10 parts of milk powder were mixed to obtain water-free cream (β-glucan content: 1.50%) according to the present invention. The β-glucans were found uniformly dispersed. The water-free cream of the present invention was evaluated for smoothness and flavor. The results are shown in Table 1. The resulting water-free cream had good melt in the mouth and a very good flavor.

Comparative Example 17: Production of water-free cream

[0163] Thirty-five parts of a fat and oil composition prepared in the same manner as in Example 8 except for using no β-glucans, 45 parts of sugar, 10 parts of a tasty powder, and 10 parts of milk powder were mixed to obtain water-free cream, which was evaluated for smoothness and flavor for comparison. The results are shown in Table 2.

Example 27: Production of whipped cream for cream sandwiches

[0164] A mixture of 100 parts of the fat and oil composition-8 containing β-glucans extracted from a gramineous plant which was prepared in Example 8 and 0.1 part of a monoglyceride was beaten into whipped cream having a specific gravity of 0.3. A hundred parts of sugar syrup was added, and the cream was further beaten to prepare whipped cream having a specific gravity of 0.65 β-glucan content: 2.15%), which was for cream sandwiches. The β-glucans were found uniformly dispersed. The whipped cream of the present invention was evaluated for smoothness and flavor. The results are shown in Table 1. The resulting whipped cream had a very good flavor.

Comparative Example 18: Production of whipped cream for cream sandwiches

[0165] Whipped cream for cream sandwiches was made in the same manner as in Example 27, except for using 100 parts of a fat and oil composition prepared in the same manner as in Example 8 except for using no β-glucans. The resulting whipped cream was evaluated for smoothness and flavor for comparison. The results obtained are shown in Table 2.

Example 28: Production of hard candy

[0166] Thirty-five parts of a fat and oil composition consisting of 100 parts of the fat and oil of Example 1, 100 parts of the fat and oil of Example 2, 23 parts of a polyglycerol fatty acid ester, 14 parts of a glycerol fatty acid ester, and 4 parts of a sucrose fatty acid ester, 35 parts of sugar, 8.5 parts of thick malt syrup, 1.5 parts of defatted milk powder, and 40 parts of water were mixed into an oil-in-water emulsion, which was boiled down until the temperature reached 140°C and further concentrated until the water content was reduced to 1.9%. The resulting thick syrup was cooled and molded to obtain hard candy (β-glucan content: 17.80%). The β-glucans were found uniformly dispersed. The hard candy of the present invention was evaluated for smoothness and flavor. The results are shown in Table 1. The resulting hard candy underwent no bleeding of oily components during storage and had a good flavor.

Comparative Example 19: Production of hard candy

[0167] Thirty-five parts of a fat and oil composition consisting of 100 parts of soybean oil, 100 parts of palm oil, 23 parts of a polyglycerol fatty acid ester, 14 parts of a glycerol fatty acid ester, and 4 parts of a sucrose fatty acid ester, 35 parts of sugar, 8.5 parts of thick malt syrup, 1.5 parts of defatted milk powder, and 40 parts of water were mixed into an oil-in-water emulsion, which was boiled down until the temperature reached 140°C and further concentrated to water content of 1.9%. The resulting thick syrup was cooled and molded to obtain hard candy. The resulting hard candy was evaluated for smoothness and flavor for comparison. The results are shown in Table 2.

Example 29: Production of whipped cream

[0168] In 50 parts of water heated to 60°C were dissolved 5 parts of defatted milk powder and 0.1 part of sodium tripolyphosphate while stirring to prepare an aqueous phase. Separately, 10 parts of the fat and oil composition of Example 1, 20 parts of the fat and oil composition of Example 2, and 15 parts of the fat and oil composition of Example 3 were mixed to prepare an oily phase. The oily phase was mixed with the aqueous phase by stirring to prepare a preliminary emulsion. The preliminary emulsion was homogenized under a pressure of 5 MPa, sterilized in a VTIS sterilization apparatus at 142°C for 4 seconds, re-homogenized under a pressure of 5 MPa, cooled to 5°C, and then

aged in a refrigerator for 24 hours to give whipped cream (β-glucan content: 19.73%) according to the present invention. The β-glucans were found uniformly dispersed. The whipped cream of the present invention was evaluated for stability, smoothness, and flavor. The results are shown in Table 1. The resulting whipped cream was proved to have satisfactory qualities of overrun, emulsion stability, heat resistant shape retention, flavor, melt-in-the-mouth, and shapability in piping into rosettes.

Comparative Example 20: Production of whipped cream

[0169] In 50 parts of water heated to 60°C were dissolved 5 parts of defatted milk powder and 0.1 part of sodium tripolyphosphate while stirring to prepare an aqueous phase. Separately, 10 parts of soybean oil, 20 parts of palm oil, and 15 parts of rapeseed oil were mixed to prepare an oily phase. The oily phase was mixed with the aqueous phase by stirring to prepare a preliminary emulsion. The preliminary emulsion was further processed in the same manner as in Example 29 to obtain whipped cream, which was evaluated for stability, smoothness, and flavor for comparison. The results obtained are shown in Table 2.

Example 30: Production of milk substitute composition

[0170] In 64 parts of water heated to 60°C were dissolved 25 parts of defatted milk powder, 0.2 part of sodium hexametaphosphate, 0.2 part of sodium citrate, and 0.3 part of a sucrose fatty acid ester while stirring to prepare an aqueous phase. To the aqueous phase was added 10 parts of the fat and oil composition of Example 6, 0.3 part of a glycerol fatty acid ester and mixed by stirring to prepare a preliminary emulsion. The preliminary emulsion was homogenized under a pressure of 5 MPa, sterilized in a VTIS sterilization apparatus at 142°C for 4 seconds, re-homogenized under a pressure of 15 MPa, and cooled to 5°C to obtain a milk substitute composition (β-glucan content: 0.09%) according to the present invention. The β-glucans were found uniformly dispersed. The milk substitute composition of the present invention was evaluated for stability and flavor. The results are shown in Table 1. The resulting milk substitute composition was proved satisfactory in both flavor and emulsion stability.

Comparative Example 21: Production of milk substitute composition

[0171] In 64 parts of water heated to 60°C were dissolved 25 parts of defatted milk powder, 0.2 part of sodium hexametaphosphate, 0.2 part of sodium citrate, and 0.3 part of a sucrose fatty acid ester while stirring to prepare an aqueous phase. To the aqueous phase were added 10 parts of the fat and oil composition of Comparative Example 6 and 0.3 part of a glycerol fatty acid ester and mixed by stirring to prepare a preliminary emulsion. The preliminary emulsion was further processed in the same manner as in Example 30 to give a milk substitute composition. The resulting milk substitute composition was evaluated for stability and flavor for comparison. The results are shown in Table 2.

Example 31: Production of food (margarine) prophylactic for habitual disease

[0172] Ten parts of hydrogenated soybean oil (melting point: 45°C), 35 parts of palm oil, 10 parts of the fat and oil composition-1 containing β-glucans extracted from a gramineous plant which was obtained in example 1, 30 parts of an interesterified oil containing 10% or more of plant sterol or plant sterol fatty acid esters, 13.3 parts of sample 1 obtained in Preparation Example 2, 1 part of edible salt, 0.5 part of defatted milk powder, and 0.2 part of flavor were emulsified and rapidly cooled for plasticization to produce margarine having a cholesterol lowering effect (β-glucan content: 4.95%) according to the present invention. The β-glucans were found uniformly dispersed. The margarine having a cholesterol lowering effect according to the present invention was evaluated for smoothness and flavor. The results are shown in Table 1. The resulting margarine had good melt in the mouth and a good flavor.

Comparative Example 22: Production of food (margarine) prophylactic for habitual disease

[0173] Ten parts of hydrogenated soybean oil (melting point: 45°C), 35 parts of palm oil, 10 parts of soybean oil, 30 parts of an interesterified oil containing 10% or more of plant sterol or plant sterol fatty acid esters, 13.3 parts of water, 1 part of edible salt, 0.5 part of defatted milk powder, and 0.2 part of flavor were emulsified and rapidly cooled for plasticization to produce margarine having a cholesterol lowering effect, which was evaluated for smoothness and flavor for comparison. The results are shown in Table 2.

Example 32: Production of drug prophylactic for habitual diseases

[0174] Three parts of high purity DHA (purity: 98%; POV: 1.0 meq/kg or less) containing 4000 ppm of α-tocopherol,

20 parts of sample 1 obtained in Preparation Example 2, and 10 parts of casein sodium were emulsified in a high-speed mixer in a nitrogen atmosphere and spray dried to prepare a powdered drug prophylactic for habitual diseases (β-glucan content: 4.60%) according to the present invention. The β-glucans were found uniformly dispersed. The drug prophylactic for habitual diseases according to the present invention was evaluated for stability. The results are shown in Table 1. The POV of the powder was 0.8 meq/kg, proving the drug to be excellent in antioxidation stability.

Comparative Example 23: Production of drug prophylactic for habitual diseases

[0175] Three parts of high purity DHA (purity: 98%; POV: 1.0 meq/kg or less) containing 4000 ppm of α-tocopherol, 20 parts of water, and 10 parts of casein sodium were emulsified in a high-speed mixer in a nitrogen atmosphere and spray dried into powder, which was evaluated for stability for comparison. The results are shown in Table 2. The POV of the powder was 1.4 meq/kg, indicating inferior antioxidation stability.

TABLE 1

Example No.	Stability	Texture		
		Smoothness	Hardness	Flavor
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	-	A	-	A
9	A	A	-	A
10	A	-	-	A
11	A	-	-	A
12	A	A	-	A
13	A	A	-	A
14	A	A	-	A
15	-	-	-	-
16	-	-	A	A
17	-	-	A	A
18	-	A	A	A
19	-	A	A	A
20	-	-	A	A
21	-	-	A	A
22	-	-	A	-
23	-	A	-	-
24	-	A	-	A
25	-	A	A	A
26	-	A	-	A
27	-	A	-	A
28	A	A	-	A

(continued)

Example No.	Stability	Texture		
		Smoothness	Hardness	Flavor
29	A	A	-	A
30	A	-	-	A
31	-	A	-	A
32	A	-	-	-

TABLE 2

Comparative Example No.	Stability	Texture		
		Smoothness	Hardness	Flavor
1	-	B	-	C
2	A	A	-	B
3	B	-	-	B
4	B	-	-	A
5	C	A	-	B
6	C	B	-	B
7	B	A	-	B
8	-	-	B	B
9	-	-	B	B
10	-	A	B	B
11	-	A	B	B
12	-	-	B	A
13	-	-	B	A
14	-	-	B	-
15	-	A	-	B
16	-	A	B	C
17	-	A	-	C
18	-	A	-	C
19	B	A	-	B
20	B	-	-	C
21	B	-	-	B
22	-	B	-	C
23	C	-	-	-

Examples 33 to 37 and Comparative Examples 24 to 27:

[0176] These Examples and Comparative Examples were carried out with the β-glucan extract (sample 2) which was prepared in Preparation Example 3 (Preparation of β-glucans).

Example 33:

[0177] In 50.5% of warm water (60°C) was dispersed 0.5% of the β-glucan extract (sample 2), and 49% of palm oil at 60°C was dispersed and emulsified therein. The resulting emulsion was homogenized at 60°C under a pressure of 100 kg/cm^2 and heated up to 100°C by use of a scraped surface heat exchanger (rotational speed: 1200 rpm). The emulsion was cooled to 5°C and aged for 24 hours to prepare an oil-in-water emulsified composition. When the resulting oil-in-water emulsified composition was stirred in a mixer, heated to 60°C, and centrifuged at a rotational speed of 3000 rpm for 20 minutes, it underwent no oil separation, showing high emulsion stability against both physical and thermal stresses.

Example 34:

[0178] In 49.5% of warm water (60°C) having dissolved therein 1% of a whey protein concentrate (mainly comprising β-lactalbumin and α-lactalbumin; protein content: 80%) was dispersed 0.5% of the β-glucan extract (sample 2), and 49% of palm oil heated at 60°C was dispersed and emulsified therein. The resulting emulsion was homogenized at 60°C under a pressure of 100 kg/cm^2 and heated up to 100°C by use of a scraped surface heat exchanger (rotational speed: 1200 rpm). The emulsion was cooled to 5°C and aged for 24 hours to prepare an oil-in-water emulsified composition. When the resulting oil-in-water emulsified composition was stirred in a mixer, heated to 60°C, and centrifuged at a rotational speed of 3000 rpm for 20 minutes, it underwent no oil separation, showing high emulsion stability against both physical and thermal stresses.

Comparative Example 24:

[0179] An oil-in-water emulsified composition was prepared in the same manner as in Example 33, except that the β-glucan extract (sample 2) as used in Example 33 was not added and that the warm water was used in a proportion of 51%. When the resulting oil-in-water emulsified composition was stirred in a mixer under the same condition as in Example 33, the oily component separated. When it was heated to 60°C and centrifuged at 3000 rpm for 20 minutes, phase separation showing marked oil separation occurred. Emulsion stability against physical and thermal stresses was not observed.

Comparative Example 25:

[0180] An oil-in-water emulsified composition was prepared in the same manner as in Example 33, except for adding 0.5% of a sucrose fatty acid ester (HLB: 16) in place of the β-glucan extract (sample 2) used in Example 33. The composition was evaluated for emulsion stability in the same manner as in Example 33. When the resulting oil-in-water emulsified composition was stirred in a mixer, heated to 60°C, and centrifuged at 3000 rpm for 20 minutes, it underwent appreciable oil separation, showing no emulsion stability against physical and thermal stresses.

Comparative Example 26:

[0181] An oil-in-water emulsified composition was prepared in the same manner as in Example 33, except for adding 0.5% of a glycerol fatty acid ester (HLB: 4.3) in place of the β-glucan extract (sample 2) used in Example 33. The composition was evaluated for emulsion stability in the same manner as in Example 33. When the resulting oil-in-water emulsified composition was stirred in a mixer, heated to 60°C, and centrifuged at 3000 rpm for 20 minutes, it underwent appreciable oil separation, showing no emulsion stability against physical and thermal stresses.

Example 35:

[0182] In 56.6% of warm water (60°C) was dispersed 0.4% of the β-glucan extract (sample 2), and 43% of palm oil heated at 60°C was dispersed and emulsified therein. The resulting emulsion was homogenized at 60°C under a pressure of 100 kg/cm^2 and heated up to 100°C by use of a scraped surface heat exchanger (rotational speed: 1200 rpm). The emulsion was cooled to 5°C and aged for 24 hours to prepare an oil-in-water emulsified composition. The resulting oil-in-water emulsified composition was frozen at -20°C for 24 hours. When the frozen oil-in-water emulsified composition was thawed at 15°C, oil separation did not occur, proving the composition to have high emulsion stability against freezing.

Comparative Example 27:

[0183] An oil-in-water emulsified composition was prepared in the same manner as in Example 35, except that the β-glucan extract (sample 2) as used in Example 35 was not added and that the warm water was used in a proportion of

57%. The resulting oil-in-water emulsified composition was frozen under the same conditions as in Example 35. When the frozen oil-in-water emulsified composition was thawed at 15°C, it underwent oil separation, showing no emulsion stability against freezing.

Example 36:

[0184] Salted egg yolk (edible salt content: 8%) was adjusted to pH 8.4 with sodium hydroxide. To 100 parts of the salted egg yolk was added 0.015 part of phospholipase A of swine pancreatic juice origin, and the mixture was treated at 40°C for 6 hours. Then 0.001 part of bromelain was added and allowed to react at 45°C for 5 hours, followed by cooling to 10°C to obtain enzyme-processed egg yolk having a water content of 46%. Water (41%), 10% of thick malt syrup (water content: 30%), 7% of vinegar (acetic acid acidity: 10%; water content: 90%), 1.8% of edible salt, 0.1% of sodium glutamate, 0.1% of animal protein hydrolyzate, 0.5% of mustard powder, and 10% of the enzyme-processed egg yolk were mixed up to prepare an aqueous phase. Separately, an oily phase was prepared by mixing 27% of soybean salad oil, 1.5% of the β-glucan extract (sample 2), and 1% of modified starch obtained by phosphoric acid-crosslinking waxy corn followed by gelatinization. The oily phase was added to the aqueous phase while stirring to obtain an oil-in-water preliminary emulsion, which was emulsified in a colloid mill to obtain a sour oil-in-water emulsion containing the β-glucans of the present invention and having a water content of 55%. A 50 g portion of the sour oil-in-water emulsion was put into a 100 ml beaker and heated in a microwave oven (high-frequency output: 500 W) for 30 seconds, and the appearance, texture, and flavor were examined. It was found as a result that the emulsion underwent no oil separation, exhibited good shape retention, and had a creamy texture and a good mayonnaise flavor. Further, another 50 g portion of the sour oil-in-water emulsion was put into a 100 ml beaker, preserved in a freezer at -20°C for 30 days, and heated as frozen in a microwave oven (high-frequency output: 500 W) for 30 seconds, and the appearance, texture, and flavor were investigated. It was found as a result that the emulsion showed no oil separation, exhibited good shape retention, and had a creamy texture and a satisfactory mayonnaise flavor.

Example 37:

[0185] A mixed oil (81.3%) consisting of 80% of hydrogenated fish oil having a melting point of 34°C, 15% of soybean oil, and 5% of soft palm oil (melting point: 25°C) was mixed with 0.1% of lecithin and 0.1% of the β-glucan extract (sample 2), and the mixture was heated to about 60°C to prepare an oily phase. Separately, an aqueous phase was prepared from 16% of water, 1% of defatted milk powder, 1% of an enzyme-processed egg yolk prepared in the same manner as in Example 36, and 0.5% of edible salt. The aqueous phase was slowly added to the oily phase while stirring thoroughly to make a water-in-oil emulsion, which was sterilized and rapidly cooled for plasticization in a conventional manner to obtain margarine for dough and batter. The resulting margarine for dough and batter was evaluated for emulsion stability (whether water was released when aged at 5°C for 24 hours and then pressed with a spatula) and flavor. As a result, water separation was not observed, and the flavor was good.

Examples 38 to 42 and Comparative Example 28:

[0186] Quality modifiers were prepared by using the β-glucan extract (sample 2) obtained in Preparation Example 3 (Preparation of β-glucans). Ingredients were compounded according to the formulation shown in Table 3 below and mixed in a 10 liter rocking mixer, a dry powder blender, for 1 hour to obtain modifiers-1 to -5 and comparative product-1 having the ingredients uniformly dispersed.

[0187] The resulting modifiers-1 to -5 and comparative product-1 were subjected to a breadmaking test (Examples 38 to 42 and Comparative Example 28). The formulations and the steps used in the test are shown in Tables 4 and 5, respectively, and the test results are shown in Table 6.

[0188] Evaluation for the properties of dough and the quality of crumb and crust, texture, softness, and flavor of bread was made according to the following standards.

1) Dough properties

A ... Not sticky
B ... Slightly sticky

2) Crumb quality - observed with the naked eye

A ... Fine cells
B ... Having slightly rough cells

3) Crust quality - observed with the naked eye

A ... Free from cracks, bumps, and roughness
B ... Slight cracks, bumps or roughness observed

4) Texture
The texture was evaluated by 10 panel members and rated A or B according to the standard shown below. The rating given by the greater number out of 10 panel members was the rating of the sample.

A ... Very good texture
B ... Good texture

5) Softness
The softness was evaluated by 10 panel members and rated A or B according to the standard shown below. The rating given by the greater number out of 10 panel members was the rating of the sample.

A ... Very soft
B ... Soft

6) Flavor
The flavor was evaluated by 10 panel members and rated A or B according to the standard shown below. The rating given by the greater number out of 10 panel members was the rating of the sample.

A ... Very good flavor
B ... Good flavor

TABLE 3

Formulation of Quality Modifier (general dough)						
	Modifier					Comparative Product 1
	1	2	3	4	5	
Extracted β-glucan	85	70	60	80	90	
Diacetyltartaric acid monoglyceride Na salt		10	15		8	20
Sucrose fatty acid ester			1			12
Fatty acid monoglyceride						8
L-Ascorbic acid		0.2	0.3	0.7		0.7
L-Cystine			0.1			0.2
α-Amylase		0.5		0.7	0.4	1
Glucoamylase						0.5
Hemicellulase		0.5			0.2	0.5
Protease						0.1
Wheat flour	15	15	21.6	16.6		54
Sugar		3.8	2	2	1.4	3
Total (%)	100	100	100	100	100	100

TABLE4

Formulation for Breadmaking by Sponge-Dough Process (unit: part)						
	Example					Comparative Example 28
	38	39	40	41	42	
Bread flour (Eagle)	70	70	70	70	70	70
Yeast	2	2	2	2	2	2
Water	46	46	46	46	46	46
Bread flour	30	30	30	30	30	30
Sugar	4	4	4	4	4	4
Edible salt	2	2	2	2	2	2
Shortening	4	4	4	4	4	4
Defatted milk powder	2	2	2	2	2	2
Modifier-1	2					
Modifier-2		2				
Modifier-3			2			
Modifier-4				2		
Modifier-5					2	
Comparative product- 1						2
Water	25	24	24	25	25	20

TABLE 5

Steps of 70% Sponge-Dough Process		
Sponge	Mixing	Mixer: Shinagawa, Type 5 DM, L: 141 rpm; H: 2385 rpm 24°C; 15 mins. at low speed; 2 mins. at high speed
	Fermentation	28°C, 30 mins.
Dough	Mixing	28°C; 1.5 mins. at low speed; 2 mins. at high speed
Floor time		28°C, 30 mins.
Shaping	Dividing	Fold into three and divided into 450 g portions
	Rounding	The dough was passed through National Sheeter-moulder (roll clearance: 3/8 in.), folded into three, passed through the sheeter, again folded into three and again sheeted.
	First proofing	28°C, 15 mins.
	Shaping	The dough was passed through the sheeter section of National Sheeter-moulder once at a roll clearance of 3/8 in. and then once at 7/32 in. in the same direction and finally at 1/8 in. in the opposite direction. The flattened dough was rolled up in the moulder section from its dry end making 15 turns.
	Shape	One-loaf
	Pan	One-loaf pan (top: 9.8 x 20.5 cm; bottom: 8.5 x 18.9 cm; 8.0 cm deep; 1445 cc)
	Final proofing Final proofing	38°C, >90% RH, until the dough rose 2.0 cm above the lip of the pan

(continued)

Steps of 70% Sponge-Dough Process		
Baking	Baking	227 to 280°C, 23 mins.
	Cooling	Room temperature, 40 mins.; sealed into a polyethylene bag after cooling

TABLE 6

	Examples					Comparative Example 28
	38	39	40	41	42	
Water absorption (%)	71	70	70	71	71	66
Dough properties	A	A	B	B	B	B
Volume (ml)	2640	2658	2620	2651	2638	2530
Crumb	A	A	A	A	A	A
Crust	B	A	B	B	B	B
Texture	A	A	A	A	B	A
Softness	A	A	B	B	A	B
Flavor	A	A	A	A	A	B

[0189] As is apparent from the test results shown in Table 6, the modifiers-1 to -5 containing the extracted β-glucans (Examples 38 to 42) manifest excellent bread quality improving effects compared with the comparative product-1 containing no β-glucans (Comparative Example 28). It is also seen that the modifiers-1 to -5 contribute to flavor enhancement and enable reduction of amounts of additives conventionally used in breadmaking without while retaining the bread quality (Examples 39 to 42).

Examples 43 to 47 and Comparative Example 29:

[0190] Quality modifiers were prepared by using the β-glucan extract (sample 2) obtained in Preparation Example 3 (Preparation of β-glucans).

[0191] Ingredients were compounded according to the formulation shown in Table 7 below and mixed in a 10 liter rocking mixer, a dry powder blender, for 1 hour to obtain modifiers-6 to -10 and comparative product-2 having the ingredients uniformly dispersed.

[0192] Frozen dough for butter rolls was prepared by using the modifiers-6 to -10 and the comparative product-2 and evaluated for breadmaking properties (Examples 43 to 47 and Comparative Example 29). The formulation in the test is shown in Table 8 below. The dough was prepared as follows. Ingredients other than the modifier and margarine were put into a mixer and mixed at a low speed for 2 minutes and at a medium speed for 5 minutes. The modifier and margarine were added, and mixing was continued at a low speed for 2 minutes and then at a medium speed for 4 minutes. The mixing temperature was 27°C. The dough was allowed to ferment at 28°C and 75% humidity for 30 minutes. The proofed dough was divided into 45 g portions. After a bench time of 15 minutes, the proofed dough was shaped, deep frozen at -38°C for 15 minutes, and stored in a freezer at -20°C. After 2, 4, 6 or 8 week storage, the frozen dough was thawed at 20°C, finally proofed at 38°C and 80% humidity, and baked in an oven at 200°C for 10 minutes to obtain butter rolls. After cooling, the butter rolls were packed in a polyethylene bag. After left to stand at 25°C for 24 hours, the butter rolls were evaluated for specific volume, hardness, appearance, and taste. The results obtained are shown in Table 9 below.

[0193] The specific volume is a value obtained by dividing the volume of a sample by its weight. The higher the value, the higher the volume. The hardness was represented by the number of grams required for 40% compressing a 3 cm thick cut piece of a sample with a disk of 2.5 cm in diameter as measured with a rheometer (Yamaden Incorporation). The smaller the number, the softer. The appearance was observed with the naked eye and ranked A to C (A: even brown color with a sheen; B: slightly uneven brown color with a poor sheen; C: considerably uneven brown color with no sheen). The taste was ranked A to C (A: excellent in flavor and softness; B: normal; C: lacking in flavor or softness).

TABLE 7

Formulation of Quality Modifier (frozen dough)						
	Modifier					Comparative Product 2
	6	7	8	9	10	
Extracted β-glucans	85	73	60	80	87	
Diacetyltartaric acid monoglyceride Na salt		15	18		8	20
Sucrose fatty acid ester			1			15
Fatty acid monoglyceride						15
L-Ascorbic acid		0.7	0.5	1		1
L-Cystine			0.2			1
α-Amylase		0.5		0.5	0.3	0.6
Glucoamylase		0.2			0.1	0.3
Hemicellulase		0.4		0.4	0.2	0.4
Protease						0.2
Activated wheat gluten	8	9.2	15	6	4.4	20
Wheat flour	7		3.3	10.1		24
Sugar		1	2	2		2.5
Total (%)	100	100	100	100	100	100

TABLE 8

Formulation of Frozen Dough (unit: part)						
	Example					Comparative Example 29
	43	44	45	46	47	
Bread flour	100	70	70	70	70	70
Yeast	4	2	2	2	2	2
Yeast food	0.1					
Water	44	46	46	46	46	46
Soft white sugar	12	4	4	4	4	4
Whole egg	10					
Edible salt	1.3	2	2	2	2	2
Margarine	10	4	4	4	4	4
Defatted milk powder	2.5	2	2	2	2	2
Modifier-6	4					
Modifier-7		4				
Modifier-8			4			
Modifier-9				4		
Modifier-10					4	
Comparative Product-2						4

TABLE 9

Evaluation for Breadmaking Properties of Frozen Dough							
		Example					Comparative Example 29
		43	44	45	46	47	
2 wks.	Specific volume	5.4	5.5	5.3	5.4	5.5	5.1
	Hardness	68	67	69	69	71	81
	Appearance	A	A	A	A	A	A
	Taste	A	A	A	A	A	B
4 wks.	Specific volume	5.1	5.5	5.3	5.2	5.4	4.8
	Hardness	73	70	75	71	77	89
	Appearance	A	A	A	A	A	A
	Taste	A	A	A	A	A	B
6 wks.	Specific volume	5.1	5.1	5.2	5.1	4.9	4.5
	Hardness	79	78	80	82	81	92
	Appearance	A	A	A	A	B	B
	Taste	A	A	A	A	B	C
8 wks.	Specific volume	4.6	4.9	5	4.8	4.7	4.4
	Hardness	85	79	82	85	87	110
	Appearance	A	A	A	A	B	B
	Taste	A	A	A	B	B	C

[0194] As is apparent from the results of evaluation shown in Table 9 above, the frozen dough containing the modifiers-6 to -10 containing the β-glucan extract and having been stored in a freezer for 2 to 8 weeks exhibited excellent breadmaking properties. It was proved that the modifiers are contributory to flavor enhancement and enable reduction of amounts of additives conventionally employed in bakery products while retaining breadmaking properties of dough.

Examples 48 to 53 and Comparative Examples 30 to 35:

[0195] Quality modifiers were prepared by using the β-glucan extract (sample 2) obtained in Preparation Example 3 (Preparation of β-glucans).

[0196] Quality modifying compositions for bakery products comprising a β-glucan extract, a glucide having at least one of an 1-2-α-D-glucopyranose bond, an 1-3-α-D-glucopyranose bond, an 14-α-D-glucopyranose bond, and an 1-6-α-D-glucopyranose bond (α-glucan), and D-fructose (Examples 48 to 53) were evaluated for volume, flavor, texture, and crispness. The results obtained are shown in Table 10 below.

[0197] Bread, French bread, Danish pastries, pizza crusts and biscuits were made in usual manners. The volume was evaluated with the naked eye. The flavor, texture, and crispness were evaluated by 10 panel members and rated on an A-to-C scale according to the following standards. The rating given to a sample by the greatest number out of the 10 panel members was the rating of the sample. For comparison, Comparative Examples 30 to 35 were carried out. In Table 10 below, the amounts of the extracted β-glucans, D-fructose, and α-glucans are represented as a percentage to wheat flour taken as 100%.

1) Volume

A ... Excellent volume

B ... Good volume

C ... Poor volume

2) Flavor

A ... Very good flavor
B ... Good flavor
C ... Poor flavor

3) Texture

A ... Very good texture
B ... Good texture
C ... Poor texture

4) Crispness

A ... Very crispy
B ... Crispy
C ... Not crispy

TABLE 10

	Example						Comparative Example					
	48	49	50	51	52	53	30	31	32	33	34	35
Bakery product	bread	French bread	Danish pastry	pizza crust	bread	biscuits	bread	French bread	Danish pastry	pizza crust	bread	biscuits
Process	straight	-	-	-	sponge-dough	-	straight	-	-	-	sponge-dough	-
Extracted β-glucans (%)	13.13	0.148	2.96	27.54	18.75	4.86	-	-	-	-	-	-
D-Fructose (%)	1.3	0.01	0.6	3.5	2.3	25	1.3	0.01	0.6	3.5	2.3	25
α-Glucan (starch) content in flour (%)	75	72	74	76	73	76	75	72	74	76	73	76
α-Glucans added (%)	0	2	0	5	2	5	0	2	0	5	2	5
Total α-glucans (%)	75	74	74	81	75	81	75	74	74	81	75	81
Extracted β-glucans/D-fructose	10.1	14.8	4.9	7.9	8.2	0.19	-	-	-	-	-	-
Extracted β-glucans/ total α-glucans	0.175	0.002	0.04	0.34	0.25	0.06	-	-	-	-	-	-
Yeast	used	used	used	used	used	not used	used	used	used	not used	used	not used
Volume	B	B	A	B	A	A	C	C	C	C	C	C
Flavor	B	A	A	B	A	B	C	C	C	C	C	C

(continued)

	Example						Comparative Example					
	48	49	50	51	52	53	30	31	32	33	34	35
Texture	B	B	B	A	A	A	C	C	C	C	C	C
Crispness	-	-	-	A	-	A	-	-	-	C	-	C

Example 54: Production of instant powdered consomme

[0198] The β-glucan extract (sample 2) obtained in Preparation Example 3 (Production of β-glucans) was mixed into a composition consisting of powdered soy sauce, edible salt, sugar, sodium glutamate, ginger powder, onion powder, fat powder, ground pepper, and beef-flavored powdered soup in such an amount as to give a 2% concentration when mixed with hot water to obtain instant powdered consomme. On pouring hot water into the instant powdered consomme, the powder was dispersed in hot water without forming lumps to give consomme containing β-glucans.

Example 55: Making of corn potage

[0199] A hundred milliliters of two-fold concentrate potage (available from Asahi Denka Kogyo K.K.), 100 ml of milk, and 20 g of the β-glucan extract (sample 2) obtained in Preparation Example 3 (Production of β-glucans) were mixed by stirring at 80°C to prepare β-glucan-containing potage. The corn potage of the present invention was excellent with no strange feeling in taste and texture, such as rough texture.

Example 56: Production of fermented lactic acid beverage

[0200] Fifteen grams of fermented milk having a milk solid content of 21%, 13 g of fructose/glucose liquid sugar, 0.5 g of pectin, 0.08 g of citric acid, 0.15 g of flavor, 2 g of the β-glucan extract (sample 2) obtained in Preparation Example 3 (Production of β-glucans), and 64 g of water were mixed by stirring to prepare a β-glucan-containing fermented lactic acid beverage. The fermented lactic acid beverage was excellent in uniformity, taste and texture.

Example 57: Making of milk beverage

[0201] One gram of the β-glucan extract (sample 2) obtained in Preparation Example 3 (Production of β-glucans) was mixed into 150 ml of milk by stirring to obtain a β-glucan-containing milk beverage. The β-glucans were found uniformly dissolved and dispersed in the beverage, and the milk beverage was excellent in taste and texture.

Example 58: Making of cocoa

[0202] The β-glucan extract (sample 2) obtained in Preparation Example 3 (Production of β-glucans) was mixed into a commercially available instant cocoa mix in such an amount as to give a 5% concentration when mixed with hot water to prepare a β-glucan-containing instant cocoa mix. On pouring hot water, the instant cocoa mix was dispersed without forming lumps to provide β-glucan-containing cocoa.

Example 59: Making of cream soup with onion

[0203] β-Glucan-containing powdered soup mix was prepared by uniformly mixing well 28.7 g of edible oil, 19 g of wheat flour, 12.52 g of dried onion chips, 12 g of defatted milk powder, 8 g of edible salt, 4 g of onion powder, 1.25 g of sodium glutamate, 2 g of chicken extract, 0.1 g of white pepper, 10 g of maize flour, 0.4 g of carrageenan, and 2.03 g of the β-glucan extract (sample 2) obtained in Preparation Example 3 (Production of β-glucans). A comparative soup mix was prepared by replacing the β-glucans with 2.03 g of wheat flour. On pouring 100 ml of hot water into 20 g of each soup mix, the β-glucan-containing soup mix was dissolved and dispersed uniformly in several seconds, whereas the comparative soup mix formed lumps and was difficult to dissolve.

Example 60: Making of *tempura* - 1

[0204] A hundred grams of commercial cake flour (Nippon Flour Mills Co., Ltd.), one whole egg, 3 g of the β-glucan extract (sample 2) obtained in Preparation Example 3 (Production of β-glucans), and 120 g of water were lightly mixed to prepare batter. Prawns from which the head and vein had been removed were each dipped in the batter and deep-dried in oil to make *tempura*. For comparison, *tempura* was made by using batter containing no β-glucans. The *tempura* of the present invention was superior to the comparative one in brown color, taste and texture.

Example 61: Making of *tempura* - 2

[0205] A hundred grams of commercial cake flour (Nippon Flour Mills Co., Ltd.), one whole egg, 3 g of the β-glucan extract (sample 2) obtained in Preparation Example 3 (Production of β-glucans), and 120 g of water were lightly mixed to prepare batter. Prawns from which the head and vein had been removed were each dipped in the batter and deep

frozen at -20°C. The resulting frozen prawn *tempura* ready to deep-fry was preserved at -18°C for 1 week and deep-dried in oil. For comparison, *tempura* was made by using batter containing no β-glucans. The *tempura* of the present invention was superior to the comparative one in brown color, taste and texture.

Example 62: Making of *okonomi-yaki* (as-you-like-it pancake)

[0206] A hundred fifty grams of commercial cake flour (Nippon Flour Mills Co., Ltd.), 3/4 cup of water, 5 g of the β-glucan extract (sample 2) obtained in Preparation Example 3 (Production of β-glucans), and a pinch of edible salt were mixed up thoroughly, and one whole egg was mixed therein to prepare thin dough. A portion of the dough was spread on a hot plate oiled with the fat and oil composition containing the β-glucans extracted from a gramineous plant which was obtained in Example 4. Cabbage, green onion, *sakura* shrimps, and green seaweed flakes were placed thereon, and the rest of the dough was poured thereon. The dough with filling was turned up and further cooked to make *okonomi-yaki*. Comparative *okonomi-yaki* was made without using β-glucans. The *okonomi-yaki* of the present invention was excellent in brown color, taste, and texture.

Example 63: Production of frozen creamy crab *korokke* (Japanese style croquettes) - 1

[0207] A pan was put on fire, and 70 parts of cake flour, 70 parts of salted butter, and 80 parts of onion were sauteed in the pan. The pan was taken off the fire, and 360 parts of milk was added. The pan was put back on the fire, and the mixture was stirred well to prepare white sauce. A hundred parts of canned crab meat was mixed into 500 parts of the white sauce, and the mixture was shaped into patties each weighing 50 g. Each patty was covered with cake flour, egg, and then bread crumbs containing 5% of the β-glucan extract (sample 2) obtained in Preparation Example 3 (Production of β-glucans) and deep frozen at -30°C. After 1 month storage at -20°C, the frozen *korokke* was thawed, deep dried in salad oil heated to 180°C for 2 minutes and 30 seconds, drained on paper towel to make creamy crab *korokke*. Comparative creamy crab *korokke* was made without using the β-glucans. The creamy crab *korokke* of the present invention was superior to the comparative one in taste and texture.

Example 64: Production of frozen creamy crab *korokke* - 2

[0208] A pan was put on fire, and 70 parts of cake flour, 70 parts of salted butter, and 80 parts of onion were sauteed in the pan. The pan was taken off the fire, and 360 parts of milk was added. The pan was put back on the fire, and the mixture was stirred well to prepare white sauce. A hundred parts of canned crab meat was mixed into 500 parts of the white sauce, and the mixture was shaped into patties each weighing 50 g. Each patty was covered with cake flour, egg, and then bread crumbs containing 5% of the β-glucan extract (sample 2) obtained in Preparation Example 3 (Production of β-glucans), and deep dried in salad oil heated to 180°C for 2 minutes and 30 seconds. The resulting *korokke* was deep frozen and stored at -20°C. For comparison, frozen *korokke* was made without using the β-glucans. After 1 month storage, the frozen *korokke* was cooked in a microwave oven (500 W) for 1 minute. The creamy crab *korokke* according to the present invention was crispy, not watery, and excellent in appearance, flavor and texture as compared with the comparative one.

Industrial Applicability:

[0209] The present invention provides a β-glucan-containing fat and oil composition which supplies β-glucans having excellent bioregulatory functions without being accompanied by reductions in taste, texture, and the like.

Claims

1. A β-glucan-containing fat and oil composition **characterized by** containing β-glucans which have a molecular weight average of less than 500.000 and are extracted from a gramineous plant.

2. The β-glucan-containing fat and oil composition according to claim 1, which contains a β-glucan having at least two kinds of bonds selected from a 1-2-β-D-glucopyranose bond, a 1-3-β-D-glucopyranose bond, a 1-4-β-D-glucopyranose bond, and a 1-6-β-D-glucopyranose bond.

3. The β-glucan-containing fat and oil composition according to claim 1 or 2, which contains a β-glucan having a 1-3-β-D-glucopyranose bond and a 1-4-β-D-glucopyranose bond.

4. The β-glucan-containing fat and oil composition according to any one of claims 1 to 3, wherein said gramineous plant is barley or oats.

5. A food containing the β-glucan-containing fat and oil composition according to any one of claims 1 to 4.

6. A fat and oil emulsified composition containing the β-glucan-containing fat and oil composition according to any one of claims 1 to 4.

7. A bakery product containing the β-glucan-containing fat and oil composition according to any one of claims 1 to 4.

8. A confectionery product containing the β-glucan-containing fat and oil composition according to any one of claims 1 to 4.

9. A food having a prophylactic action for a habitual disease containing the β-glucan-containing fat and oil composition according to any one of claims 1 to 4.

10. A drug having a prophylactic action for a habitual disease containing the β-glucan-containing fat and oil composition according to any one of claims 1 to 4.

11. A processed rice, wheat, maize or soybean product containing the β-glucan-containing fat and oil composition according to any one of claims 1 to 4.

12. A liquid food containing the β-glucan-containing fat and oil composition according to any one of claims 1 to 4.

13. A processed food mainly comprising starch and containing the β-glucan-containing fat and oil composition according to any one of claims 1 to 4.

Patentansprüche

1. β-Glucan enthaltende Zusammensetzung aus Fett und Öl, **dadurch gekennzeichnet, dass** sie β-Glucane enthält, die einen Molekulargewichtsdurchschnitt von weniger als 500000 haben und aus einer Graspflanze extrahiert sind.

2. β-Glucan enthaltende Zusammensetzung aus Fett und Öl nach Anspruch 1, die ein β-Glucan mit wenigstens zwei Arten von Bindungen, ausgewählt aus einer 1-2-β-D-Glucopyranose-bindung, einer 1-3-β-D-Glucopyranosebindung, einer 1-4-β-D-Glucopyranosebindung und einer 1-6-β-D-Glucopyranose-bindung, enthält.

3. β-Glucan enthaltende Zusammensetzung aus Fett und Öl nach Anspruch 1 oder 2, die ein β-Glucan mit einer 1-3-β-D-Glucopyranosebindung und einer 1-4-β-D-Glucopyranosebindung enthält.

4. β-Glucan enthaltende Zusammensetzung aus Fett und Öl gemäß einem der Ansprüche 1 bis 3, wobei die Graspflanze Gerste oder Hafer ist.

5. Nahrung, enthaltend die β-Glucan enthaltende Zusammensetzung aus Fett und Öl gemäß einem der Ansprüche 1 bis 4.

6. Emulgierte Zusammensetzung aus Fett und Öl, enthaltend die β-Glucan enthaltende Zusammensetzung aus Fett und Öl gemäß einem der Ansprüche 1 bis 4.

7. Bäckereiprodukt, enthaltend die β-Glucan enthaltende Zusammensetzung aus Fett und Öl gemäß einem der Ansprüche 1 bis 4.

8. Süßwarenprodukt, enthaltend die β-Glucan enthaltende Zusammensetzung aus Fett und Öl gemäß einem der Ansprüche 1 bis 4.

9. Nahrung mit einer prophylaktischen Wirkung für eine Gewohnheitskrankheit (habitual disease), enthaltend die β-Glucan enthaltende Zusammensetzung aus Fett und Öl gemäß einem der Ansprüche 1 bis 4.

10. Arzneimittel mit einer prophylaktischen Wirkung für eine Gewohnheitskrankheit, enthaltend die β-Glucan enthaltende Zusammensetzung aus Fett und Öl gemäß einem der Ansprüche 1 bis 4.

11. Behandeltes Reis-, Weizen-, Mais- oder Sojabohnenprodukt, enthaltend die β-Glucan enthaltende Zusammensetzung aus Fett und Öl gemäß einem der Ansprüche 1 bis 4.

12. Flüssige Nahrung, enthaltend die β-Glucan enthaltende Zusammensetzung aus Fett und Öl gemäß einem der Ansprüche 1 bis 4.

13. Behandelte Nahrung, die hauptsächlich Stärke beinhaltet und die β-Glucan enthaltende Zusammensetzung aus Fett und Öl gemäß einem der Ansprüche 1 bis 4 enthält.

Revendications

1. Composition d'huile et de graisses contenant des β-glucanes **caractérisée en ce qu'**elle contient des β-glucanes qui ont un poids moléculaire moyen inférieur à 500 000 et qui sont extraits d'une plante graminée.

2. Composition d'huile et de graisses contenant des β-glucanes selon la revendication 1, qui contient un β-glucane ayant au moins deux types de liaisons choisies à partir d'une liaison 1-2-β-D-glucopyranose, une liaison 1-3-β-D-glucopyranose, une liaison 1-4-β-D-glucopyranose et une liaison 1-6-β-D-glucopyranose.

3. Composition d'huile et de graisses contenant des β-glucanes selon la revendication 1 ou 2, qui contient un β-glucane ayant une liaison 1-3-β-D-glucopyranose et une liaison 1-4-β-D-glucopyranose.

4. Composition d'huile et de graisses contenant des β-glucanes selon l'une quelconque des revendications 1 à 3, dans laquelle ladite plante graminée est l'orge ou l'avoine.

5. Aliment contenant la composition d'huile et de graisses contenant des β-glucanes selon l'une quelconque des revendications 1 à 4.

6. Composition émulsifiée d'huile et de graisses contenant la composition d'huile et de graisses contenant des β-glucanes selon l'une quelconque des revendications 1 à 4.

7. Produit de boulangerie contenant la composition d'huile et de graisses contenant des β-glucanes selon l'une quelconque des revendications 1 à 4.

8. Produit de confiserie contenant la composition d'huile et de graisses contenant des β-glucanes selon l'une quelconque des revendications 1 à 4.

9. Aliment ayant une action prophylactique pour une maladie courante contenant la composition d'huile et de graisses contenant des β-glucanes selon l'une quelconque des revendications 1 à 4.

10. Médicament ayant une action prophylactique pour une maladie courante contenant la composition d'huile et de graisses contenant des β-glucanes selon l'une quelconque des revendications 1 à 4.

11. Produit traité de riz, blé, maïs ou soja contenant la composition d'huile et de graisses contenant des β-glucanes selon l'une quelconque des revendications 1 à 4.

12. Aliment liquide contenant la composition d'huile et de graisses contenant des β-glucanes selon l'une quelconque des revendications 1 à 4.

13. Aliment traité comprenant essentiellement de l'amidon et contenant la composition d'huile et de graisses contenant des β-glucanes selon l'une quelconque des revendications 1 à 4.

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(54) Oil bodies and method of producing such oil bodies

(57) The present invention relates to a process for obtaining oil bodies, said oil bodies having a volume weighted mean diameter within the range of 0.1-100 μm and comprising a lipid matrix surrounded by a layer of phospholipids embedded with structural proteins, wherein the lipid matrix contains at least 80 wt.% of glycerides, said glycerides containing at least 3 % ω-3 C_{20}-C_{24} polyunsaturated fatty acid residues (ω-3 LC PUFA) by weight of the total amount of fatty acid residues contained in said glycerides. The invention further relates to oil bodies obtainable by the process of the invention and to compositions comprising at least 0.1 wt.% of these oil bodies.

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Description

TECHNICAL FIELD OF THE INVENTION

[0001] The present invention relates to compositions comprising oil bodies. These oil bodies have a volume weighted mean diameter within the range of 0.1-100 μm and comprise a lipid matrix surrounded by a layer of phospholipids embedded with structural proteins.

[0002] The invention also concerns a novel process for the production of oil bodies.

BACKGROUND OF THE INVENTION

[0003] It is well-known in the food industry that the application of oils containing high levels of polyunsaturated fatty acids can give rise to serious off-flavour problems. These off-flavour problems are associated with the oxidation of the polyunsaturated fatty acids, leading to the formation of volatile, potent flavour molecules, such as unsaturated aldehydes. Flavour attributes associated with oxidation products of polyunsaturated fatty acids include "fish", "cardboard", "paint", "oily", "rancid" and "metallic".

[0004] Whilst all polyunsaturated fatty acids have been associated with the aforementioned stability problems, lipids containing w-3 polyunsaturated fatty acid residues, notably lipids containing ω-3 C_{20}-C_{24} polyunsaturated fatty acid residues (ω-3 long chain PUFA or ω-3 LC PUFA) are known to be extremely susceptible to oxidation. Oxidation of the latter fatty acid residues yields volatile oxidation products that introduce a foul "fishy" smell to the product containing these fatty acid residues. Typical examples of ω-3 LC PUFA include docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA).

[0005] Although it is known that the rate of oxidation of polyunsaturated fatty acids may be reduced through the addition of anti-oxidants, the application of such anti-oxidants at best delays the formation of unacceptable off-flavour. However, the shelf-life of products containing appreciable amounts of w-3 LC PUFA, especially food products and beverages, is inherently limited as a result of the inevitable oxidation of these fatty acid residues. Furthermore, in some product applications, e.g. in water-containing products containing dissolved pro-oxidative salts of e.g. iron or copper, it is essentially impossible to employ ω-3 LC PUFA as rapid oxidation of these fatty acid residues will commence as soon as the product has been compounded.

[0006] Many, many scientific publications have been issued during the past two decades that strongly suggest that regular consumption of significant amounts of ω-3 LC PUFA can deliver important health benefits. In particular, there is compelling evidence that mental health, (development of) cognitive capabilities and cardiovascular health are positively affected by the consumption of ω-3 LC PUFA. Hence, there has been no lack of effort in the industry to develop food products, beverages, nutritional preparations and pharmaceutical preparations that contain appreciable amounts of ω-3 LC PUFA. However, widespread application of ω-3 LC PUFA in especially food products and beverages has been frustrated by the oxidative instability of these fatty acids.

SUMMARY OF THE INVENTION

[0007] The present inventors have unexpectedly that the oxidative instability of ω-3 LC PUFA in products can be reduced dramatically by incorporating ω-3 LC PUFA in the form of oil bodies and that these oil bodies can be obtained with a special process.

[0008] Oil bodies are the discrete intracellular organelles in which triacylglycerides are stored in, for instance, plant seeds. Plant oil bodies are spherical particles about 0.5-4 μm in diameter and are found in all oilseed plants (i.e. canola, flax, sunflower, soybean and corn). The oil-bodies consist primarily of a central droplet of oil (predominantly consisting of triglycerides) that is surrounded by a coat essentially consisting of a phospholipid monolayer and structural proteins.

[0009] Oil bodies are remarkably stable both inside cells and in isolated preparations. In both situations the oil bodies occur as individual entities. The structural proteins that together with the phospholipid monolayer surround the central lipid core of the oil bodies, are believed to protect the phospholipids in the monolayer by making them inaccessible to external phospholipase A2 and phospholipase C. Structural proteins found in plant oil bodies include oleosins and caleosins.

[0010] The structural protein oleosin is a relatively small protein (15 to 26 kD) and typically contains a central stretch of about 72 hydrophobic residues that is inserted into the lipid core whereas the amphiphatic N-terminal and C-terminal are located at the surface of the oil body. Both terminal portions of the oleosin can be either short (e.g. 4 residues) or very long (e.g. 1200 residues). The central hydrophobic stretch of about 72 uninterrupted hydrophobic residues is the hallmark of an oleosin. No other known protein in any organism has such a long hydrophobic stretch (Hsieh et al., Plant Physiol. 2004 November; 136(3): 3427-3434).

[0011] The key structural features of caleosins are an N-terminal region with a single Ca^{2+}- binding EF hand domain,

a central hydrophobic region able to form a single bilayer span, and a C-terminal region with several putative protein kinase phosphorylation sites (D.J. Murphy, LIPID ASSOCIATED PROTEINS IN PLANTS, 16th Int. Plant Lipid Symposium, Budapest, June 2004, pages 1-8). Caleosins lack an N-terminal signal peptide, but do have a central, hydrophobic region of typically more than 30 residues. Like oleosins, caleosins contain a proline-rich region with the potential to form a "proline know" motif.

[0012] The inventors have found that ω-3 LC PUFA containing oil bodies that are naturally present in some micro-organisms, notably algae, may be recovered from these micro-organisms essentially undamaged and that the use of such oil bodies in food products provides the well-sought oxidative stability for ω-3 LC PUFA.

DETAILED DESCRIPTION OF THE INVENTION

[0013] The invention relates to a process for the isolation of oil bodies as defined herein before, said process comprising:

- subjecting micro-organisms containing such oil bodies in a dry form to conditions that will cause rupture of the micro-organism cells;
- optionally adding water, and creating a dispersion of the ruptured cells in water;
- allowing the oil bodies to be released from the cells into the surrounding water; and
- separating the oil bodies from the ruptured cell biomass.

[0014] The micro-organisms are in a dry form before they are subjected to conditions that will cause rupture of the micro-organism cells.

[0015] According to a particularly preferred embodiment, the micro-organisms employed in the present process are marine micro-organisms, preferably an algae strain belonging to one of the species *Crypthecodinium* cohnii, *Odontella aurita* or an algae strain of a species belonging to the *Thraustochytriidae* group. Most preferably, the algae strain is an algae strain of a species belonging to the *Thraustochytriidae* group.

[0016] In the present method, the oil bodies may be separated from the ruptured cell biomass by means of any suitable separation technique. Examples of suitable separation techniques include decanting, filtration and centrifugation. Most preferably, the oil bodies are separated from the ruptured cell biomass by means of centrifugation. In order to facilitate separation of the oil bodies from the ruptured cell biomass, suitably processing agents are added, e.g. to increase the density of the aqueous cell biomass.

[0017] Accordingly, one aspect of the invention relates to a composition comprising at least 0.1 wt.% of oil bodies, said oil bodies having a volume weighted mean diameter within the range of 0.1-100 μm and comprising a lipid matrix surrounded by a layer of phospholipids embedded with structural proteins, wherein the lipid matrix contains at least 80 wt.% of glycerides, said glycerides containing at least 3 %, preferably at least 5 % ω-3 C_{20}-C_{24} polyunsaturated fatty acid residues (ω-3 LC PUFA) by weight of the total amount of fatty acid residues contained in said glycerides.

[0018] The term "glyceride" as used herein encompasses lipids selected from the group consisting of triglycerides, diglycerides, monoglycerides, phosphoglycerides and combinations thereof. More preferably, the glycerides are selected from the group consisting of triglycerides and diglycerides. Most preferably, the glyceride is triglyceride oil.

[0019] The aforementioned lipid matrix represents the core of the oil body and preferably contains at least 90 wt.% of glycrerides, even more preferably at least 95 wt.% of glycerides and most preferably at least 98 wt.% of glycerides. Besides glycerides of various structures and fatty acid chain length the lipid matrix may also contain other minor components that are dissolved in said matrix.

[0020] The composition according to the present invention advantageously comprises 0.3-10 wt.% of the oil bodies. Even more preferably, said composition contains 0.5-8 wt.% of said oil bodies. The volume weighted mean diameter of the present oil bodies typically is within the range of 0.3-50 μm, especially within the range of 0.5-30 μm. The phospholipid layer surrounding the lipid core of the oil body usually is a monolayer.

[0021] The oil bodies employed in according with the present invention are advantageously derived from a micro-organism selected from the group consisting of algae, fungi, yeast and combinations thereof. More preferably, the oil bodies are derived from algae or fungi, most preferably from algae. The amino acid sequence of the structural protein in the oil bodies can be used to identify the exact origin of the oil body.

[0022] The oil bodies of the present invention are suitably isolated from marine micro-organisms. Members of the following families are capable of producing ω-3 LC PUFA: *Thraustochytriidae, Crypthecodiniaceae, Gymnodiniaceae, Prorocentraceae, Haptophyceae, Skeletonemataceae, Cryptomonadaceae, Phaeodactylaceae.* Specific examples of ω-3 LC PUFA producing species include: *Amphidinium, Amphor, Asterionella, Biddulphia, Ceratium, Chaetoceros, Chlorella, Chroomonas, Cochlodinium, Crisphaera, Crypthecodinium, Cryptomonas, Cyclotella, Cylindrotheca, Dunaiella, Emiliania, Fragilaria, Glenodinium, Gonyaulax, Gyrodinium, Haematococcus pluvialis, Heteromastix, Isochrysis, Lauderia, Monochrysis, Monodus, Moritella, Mortierella, Nannochloris, Nannochloropis, Navicula, Nitzschia, Odontella, Olisthodiscus, Pavlova, Peridinium, Phaeodactylum, Porphyridium, Prorocentrum, Pseudopedinella, Rhodella, Rho-*

dodomas, Schizochytrium, Skelentonema, Stauroneis, Tetraselmis, Thalassiosira, Thrautochytrium, Ulkenia.

[0023] According to a particularly preferred embodiment, the oil bodies are derived from marine micro-organisms, preferably from an algae strain belonging to one of the species *Crypthecodinium cohnii, Odontella aurita* or an algae strain of a species belonging to the *Thraustochytriidae* group. Most preferably, the algae strain is an algae strain of a species belonging to the *Thraustochytriidae* group. Preferred species belonging to the *Thraustochytriidae* group are *Schizochytrium, Thraustochytrium, Ulkenia, Aplanochytrium, Japonochytrium.*

[0024] Lipid containing isolates from algae have been marketed for use in animal feed. These isolates contain appreciable amounts of algae biomass. In accordance with the present invention it is preferred to employ relatively 'clean' oil bodies. Accordingly, in advantageous embodiment, no algae biomass or more preferably no biomass at all, is attached to the oil bodies or such (algae) biomass is attached in an amount of less than 50%, preferably of less than 30% by weight of the oil bodies.

[0025] The benefits of the present invention are particularly appreciated when the composition contains a significant amount of water, e.g. at least 1 wt.% of water. Preferably, the present composition contains at least 5 wt.%, more preferably at least 10 wt.% and most preferably at least 15 wt.% of water. In case the present product contains water, the oil bodies are preferably dispersed within the water.

[0026] As explained herein before, oxidation of ω-3 LC PUFA is stimulated by the presence of pro-oxidative metal salts. The oil bodies of the present invention display a remarkable oxidative stability even in the presence of such pro-oxidative metal salts. Presence of pro-oxidative metal salts in a product cannot be avoided if tap water or mineral water is used. Furthermore, the incorporation of such metals may be desirable from a nutritional perspective. Hence, according to a preferred embodiment, the water contained in the present product contains at least 5 mg per kg of water, preferably at least 10 mg per kg of water of a pro-oxidative metal cation selected from iron, copper, zinc and combinations thereof. According to a particularly preferred embodiment, the present composition contains at least 0.1 mg/kg or even at least 1 mg/kg of iron cations, more preferably at least 5 mg/kg, even more preferably at least 10 mg/kg and most preferably at least 15 mg/kg of iron cations.

[0027] Triglycerides typically constitute the bulk of the oil bodies of the present invention. Typically, the oil bodies contain at least 80 wt.% of triglycerides. More preferably, the oil bodies contain at least 90 wt.% of triglycerides. The triglycerides contained in the lipid matrix preferably contain at least 10%, most preferably at least 20% ω-3 LC PUFA by weight of the total amount of fatty acid residues contained in said triglycerides.

[0028] The oil bodies of the present invention can suitably contain a variety of ω-3 LC PUFA, including eicosapentaenoic acid and docosahexaenoic acid. The ω-3 LC PUFA in the present oil bodies is preferably selected from eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and combinations thereof. Most preferably, ω-3 LC PUFA is DHA.

[0029] In algae oil usually both EPA and DHA are present. Typically, the weight ratio of EPA:DHA in algae oil is in the range of 1:100 to 100:1. In the present oil bodies, the weight ratio of EPA:DHA preferably is in the range of 1:80 to 10:1, more preferably in the range of 1:60 to 1:10.

[0030] Phospholipids are normally present in the oil bodies of the present invention in a concentration of 0.2-5 wt.%. The structural proteins usually represent 0.2-7 wt.% of the oil bodies.

[0031] The structural protein present in the oil bodies of the present invention preferably has a hydrophilic N-terminal portion and/or a hydrophilic C-terminal portion; and a central stretch of preferably at least 25, more preferably of at least 30, even more preferably at least 50 and most preferably at least 70 uninterrupted hydrophobic residues. According to another preferred embodiment, the central stretch of hydrophobic residues of the structural protein is inserted into the lipid core and the amphiphatic N-terminal and/or amphiphatic C-terminal are located at the surface of the oil bodies, with positively charged residues embedded in a phospholipid monolayer and the negatively charged ones exposed to the exterior. In accordance with a particularly preferred embodiment, the structural protein contains both an amphiphatic N-terminal and an amphiphatic C-terminal with are both located at the surface of the oil bodies. Most preferably, the structural protein employed in accordance with the present invention is an oleosin or a caleosin.

[0032] The structural proteins present in the oil bodies of the present invention typically have a molecular size of 10-100 kD, more preferably of 12-70 kD.

[0033] The present invention enables the application of ω-3 LC PUFA in a wide array of food products. Thus, in an advantageous embodiment, the composition is an edible composition, such as a food product or a beverage. Even more preferably, the composition is selected from the group consisting of beverages (e.g. nutritional beverages and dairy drinks), spreads, nutrition bars, pasta products, ice cream, desserts, dairy products (e.g. yogurt, quark, cheese), dressings, sauces, soups, instant powders, fillings, dips and breakfast type cereal products (e.g. porridge). According to a particularly preferred embodiment, the edible product is a beverage, a nutrition bar or an instant powder. The term "instant powder" refers to an essentially dry powder that prior to consumption can be reconstituted with water to produce, for instance, a beverage or a soup.

[0034] The edible composition of the present invention may suitably contain additional ingredients such as carbohydrates, emulsifiers, herbs, spices, anti-oxidants, preservatives, flavourings, colourings, vitamins and minerals. Furthermore, the edible composition may optionally comprise, in suitable amounts, one or more agents which may beneficially

influence (post-prandial) energy metabolism and substrate utilisation, for example caffeine and flavonoids (including tea catechins, capsaicinoids and canitine).

[0035] According to a preferred embodiment, the edible composition contains added vitamins selected from at least one of: Vitamin A, Vitamin B1, Vitamin B2 (riboflavin), Vitamin B3 (Niacinamide), Vitamin B5 (d-Ca-pantothenate), Vitamin B6, Vitamin B11, Vitamin B12 (Cyanocobalamin), Vitamin C (Ascorbic acid), Vitamin D, Vitamin E (Tocopherol),Vitamin H (Biotin), and Vitamin K.

[0036] The composition also preferably comprises added minerals selected from at least one of; calcium, magnesium, potassium, zinc, iron, cobalt, nickel, copper, iodine, manganese, molybdenum, phosphorus, selenium and chromium.

[0037] One or more of the above-mentioned vitamins and minerals are preferably present at amounts of from 5 to 45% of the amounts given in the above European Commission Directive 96/8/EC, especially 5 to 40%, most especially 10 to 30%.

[0038] Another aspect of the present invention relates to the use of the oil bodies as defined herein before in the treatment or prevention of disorders or of impaired cognitive function. Examples of disorders that may suitably be treated with the present oil bodies include atherosclerosis, cardiovascular disorders and coronary heart disease. The ω-3 LC PUFA in the oil bodies helps to reduce or prevent serum oxidative stress leading to the aforementioned disorders. The present oil bodies are also advantageously used in the improvement or treatment of cognitive and mental conditions and disorders as well as the maintenance of normal functions of brain-related systems and processes, preferably ADHD, aging, Alzheimer's disease, Parkinson's disease, multiple sclerosis (MS), dyslexia, depression, learning capabilities, stress, anxiety, concentration and attention.

[0039] The aforementioned uses preferably comprise oral administration of the oil bodies. Typically, the oil bodies are administered in an amount that is sufficient to deliver at least 10 mg of ω-3 LC PUFA per administration event. More preferably, at least 30 mg, even more preferably at least 50 mg and most preferably at 100 mg of ω-3 LC PUFA is delivered per administration event.

[0040] As will be appreciated from the above, the oil bodies are advantageously ingested in the form of an edible product, e.g. a food product, a beverage, a nutritional preparation or a pharmaceutical preparation. Most preferably, the edible product is a food product or a beverage. It will be understood that the present invention may suitably be employed to treat animals, such as mammals. Most preferably, the present invention is used to treat humans.

[0041] The invention is further illustrated by means of the following examples.

EXAMPLES

Example 1

[0042] Oil bodies were extracted from dry *Schizochytrium* micro-algae biomass. The composition of the starting material is presented in Table 1:

Table 1

	Wt.%
Inorganic matter	8.2
Fat #	59.4
Protein	2.5
Moisture	11.4

(continued)

	Wt.%
Carbohydrates *	18.5
* Value estimated by subtraction of 100% minus protein, fat, inorganic matter and water content. # Soxhlet extraction of 80 grams of dried algae with hexane, followed by hexane removal in a rotary evaporator, yielded 19.2 grams of extracted oil, said oil having a fatty acid composition containing 0.05% α-linolenic acid (ALA), 1.32% EPA and 34.72% DHA.	

[0043] The oil bodies were extracted from dry algae biomass using the following procedure. Fifty gram of dry *Schizochytrium* micro-algae biomass was mixed with 500 ml of grinding solution (0.6M sucrose) in a Waring blender at a speed of 22,000 rpm for one minute. The extraction solution, which contained the oil bodies and different solutes, was separated from debris using a laboratory sieve with a mesh size of 150 μm.

[0044] The liquid phase was further processed in a Sorvall centrifuge, equipped with a GSA rotor, for 30 minutes at 8,000 rpm in order to isolate the oil bodies. A separation in three phases was observed. At the bottom a layer of sediment, consisting mainly of fibres and proteins was formed. In the middle an aqueous phase consisting of water and solutes was formed. The top layer consisted of a creamy phase consisting of oil bodies mixed with water and some impurities.

[0045] The cream top layer was carefully removed with a spoon and subjected to three more liquid-liquid separation steps successively using three different aqueous buffer solutions 1, 2 and 3.

Floating buffer 1	0.2M sucrose / 1.0M NaCl
Floating buffer 2	0.1M sucrose / 1.0M NaCl
Floating buffer 3	0.2M NaCl

[0046] The composition of the *Schizochytrium* micro-algae oil bodies was determined for three different batches of oil bodies. The results are presented in Table 2:

Table 2: Composition of three different batches of *Schizochytrium* micro-algae oil bodies.

		Batch 1	Batch 2	Batch 3
Fat (wt%)		24.9	16.1	--
Moisture (wt%)		68.9	72.8	84.7
Protein (wt%)		--	1.9	--
Particle size distribution*	$D_{3,2}$ (μm)	4.4	9.5	4.8 / 4.8
	$D_{4,3}$ (μm)	7.8	20.4	9.7 / 10.1
* $D_{3,2}$ is the surface-weighted mean diameter and $D_{4,3}$ is the volume-weighted mean diameter.				

[0047] Both the isolated oil bodies as well as the algae starting material were analysed by means of confocal scanning laser microscopy (CSLM). Microscopic observations were performed on a Bio-Rad confocal microscope equipped with a Zeiss inverted microscope system using an oil immersion 40 times objective lens N.A. 1.25. The Bio-Rad system was controlled by Lasersharp software running under OS2. Two laser lines from the argon / krypton laser, 488 and 647 nm were used to excite the fluorochrome nile blue. Green fluorescence (excitation wavelength 488 nm) indicating the

presence of lipids was detected in a band at 605 +/- 16 nm. The red fluorescence (excitation wavelength 647 nm) indicating the presence of protein and other more polar components was detected at 680 +/-16 nm.

[0048] *Schizochytrium* micro-algae biomass and the isolated oil body suspensions isolated therefrom were coloured with a nile blue solution. On both the CSLM images of the *Schizochytrium* micro-algae and the oil body suspensions, green spheres were seen. In the CSLM images of the micro-algae biomass, substantial parts of the green spheres were coloured red, indicating that protein cell-wall material is attached to the oil core.

Example 2

[0049] In order to investigate the oxidative stability of oil bodies an accelerated storage test was performed. The stability of the oil contained in oil bodies obtained through the isolation procedure described in Example 1 was compared with the oxidative stability of commercially available pure oil isolated from the same *Schizochytrium* source (ex Martek Biosciences, USA). Unlike the isolated oil bodies, the pure algae oil contained added anti-oxidants (rosemary extract, tocopherols and ascorbyl palmitate).

[0050] In a series of serum flasks were added: 16.7 gram of oil bodies of batch 1 of Example 1 or a comparable amount of the same type of pure oil (5.0 gram), 0.140 g of Hymono and 60 ml of a 0.035 wt% citric acid buffer pH 5.0. The flasks was flushed with air for five minutes to ensure that the headspace contained 20.9% oxygen. To minimise light-induced oxidation of the emulsions, the flasks were wrapped in aluminium foil, incubated in an incubator shaker (Innova 4230, New Brunswick Scientific, USA) at 60 °C, and shaken at 250 rpm for a maximum of 96 hours. At time intervals of 24 hours, oxygen headspace analysis (Servomex 574 provided with Digitron pressure indicator P200AH) was performed on headspace samples taken from the flasks.

[0051] The results obtained are depicted in Table 3:

Table 3

	Oxygen concentration after				
	0 hr	24 hrs	48 hrs	72 hrs	96 hrs
Pure oil	20.9	19.8	15.3	6.8	0.1
Oil bodies	20.9	19.9	19.2	18.6	18.1

[0052] These results clearly show that much less oxygen is consumed by sample containing the oil bodies than the by the sample containing the pure oil.

[0053] In addition, off-flavour formation after accelerated storage was analysed: Approximately 20 gram emulsion was distributed among 20 headspace vials (Chrompack, 20 ml). In all samples the amount of fat was kept constant. The sample vials were flushed with 100% oxygen, capped and placed in an oven at 60 °C in the dark. The samples are oxidised in the oven without stirring or mechanical agitation. Duplicate samples were removed from the oven at regular time intervals (typically twice a day) and cooled down to ambient temperature in the dark. The samples were de-capped and flushed for ca. 6 seconds with a nitrogen stream at a flow rate of 500 ml/min to prevent further oxidation. The samples were re-capped and stored at -18 °C until further analysis.

[0054] Samples oxidised for various lengths of time were collected for headspace analysis using a GC-17A gas chromatograph (Shimadzu). The samples were placed in an automated headspace sampler at 40 °C and allowed to equilibrate for at least 30 minutes. Headspace samples (2.5 ml) were taken with a gas-tight syringe and injected in split mode (ratio 5:1) onto a capillary CP-Sil 5CB column (Chrompack). Column specifications: length 25 m, inside diameter 0.25 mm, outside diameter 0.39 mm and film thickness 1.20 μm. The chromatograph was equipped with a flame ionisation detector. Temperatures at the inlet and detector were 200 °C and 280 °C, respectively. The temperature programming for gas chromatographic analysis was: 0 °C (4 min) - 40 °C/min - 40 °C (0 min) - 5 °C/min - 60 °C (0 min) - 40 °C/min - 200 °C (3 min). Samples were measured in random order to avoid systematic errors due to e.g. carry over effects. Peak areas (in μV.s) were quantified for the following oxidation off-flavours: acetaldehyde, propenal, pentane, 1-penten-3-one, 1-penten-3-ol, pentanal, trans-2-pentenal and hexanal. Peak areas of duplicate samples were averaged and plotted as a function of storage time at 60 °C. The results obtained are presented in Tables 4-11.

Table 4

	Acetaldehyde concentration (in μV.s) after					
	0 hr	16 hrs	24 hrs	40 hrs	48 hrs	64 hrs
Pure oil	715	3380	9837	49410	61874.5	698091

(continued)

	Acetaldehyde concentration (in μV.s) after					
	0 hr	16 hrs	24 hrs	40 hrs	48 hrs	64 hrs
Oil bodies	661.5	9038.5	8122	30573	44241.5	141770

Table 5

	Propenal concentration (in μV.s) after					
	0 hr	16 hrs	24 hrs	40 hrs	48 hrs	64 hrs
Pure oil	888	4500.5	5932.5	44065	78036.5	1573386
Oil bodies	0	2121	1994	3238.5	3221	10104

Table 6

	Pentane concentration (in μV.s) after					
	0 hr	16 hrs	24 hrs	40 hrs	48 hrs	64 hrs
Pure oil	743	395.5	564	6412	4662	198360
Oil bodies	9879.5	3824.5	5757.5	4335	3969	7940.5

Table 7

	1-Penten-3-one concentration (in μV.s) after					
	0 hr	16 hrs	24 hrs	40 hrs	48 hrs	64 hrs
Pure oil	100.5	16	9	629	648.5	2695.5
Oil bodies	13.5	18	112.5	365	372	816.0

Table 8

	1-Penten-3-ol concentration (in μV.s) after					
	0 hr	16 hrs	24 hrs	40 hrs	48 hrs	64 hrs
Pure oil	110	97	128.5	1858	3606.5	184499
Oil bodies	135.5	320.5	324	1182	1140	6274

Table 9

	Pentanal concentration (in μV.s) after					
	0 hr	16 hrs	24 hrs	40 hrs	48 hrs	64 hrs
Pure oil	2277	1839.5	2069.5	7627.5	9942.5	55320
Oil bodies	250.5	4765	1920	8671	10295	33028

Table 10

	Tr-2-pentenall concentration (in μV.s) after					
	0 hr	16 hrs	24 hrs	40 hrs	48 hrs	64 hrs
Pure oil	10	142	225.5	3364.5	5160.5	111503
Oil bodies	11.5	152	140.5	1134.5	1219.5	6615

Table 11

	Hexanal concentration (in μV.s) after					
	0 hr	16 hrs	24 hrs	40 hrs	48 hrs	64 hrs
Pure oil	799	2402.5	5633	19100.5	19100.5	266354
Oil bodies	258.5	3651.5	2080.5	2080.5	2080.5	113038

[0055] These results show clearly that the formation of secondary oxidation products is decreased in *Schizochytrium* micro-algae oil bodies in comparison to the pure algae oil. This observation is in-line with the above mentioned results from the accelerated storage test. The combined results of these tests demonstrate that *Schizochytrium* micro-algae oil bodies have a better oxidative stability than the pure algae oil. The aforementioned findings are truly remarkable since the pure algae oil contained added anti-oxidants, whereas the oil bodies did not.

Claims

1. Process for the isolation of oil bodies said oil bodies having a volume weighted mean diameter within the range of 0.1-100 μm and comprising a lipid matrix surrounded by a layer of phospholipids embedded with structural proteins, wherein the lipid matrix contains at least 80 wt.% of glycerides, said glycerides containing at least 3 %, preferably at least 5 % ω-3 C_{20}-C_{24} polyunsaturated fatty acid residues by weight of the total amount of fatty acid residues contained in said glycerides, said process comprising:

   - subjecting dry micro-organisms containing such oil bodies to conditions that will cause rupture of the micro-organism cells;
   - optionally adding water, and creating a dispersion of the ruptured cells in water;
   - allowing the oil bodies to be released from the cells into the surrounding water; and
   - separating the oil bodies from the ruptured cell biomass.

2. Process according to claim 1, wherein the micro-organism is a marine micro-organism.

3. Process according to claim 2 wherein the marine micro-organism is an algae strain selected from the group consisting of *Crypthecodinium cohnii, Odontella* aurita and *Thraustochytriidae.*

4. Process according to any of claim 1 to 3, wherein the oil bodies are separated from the ruptured cell biomass by means of centrifugation.

5. Oil bodies obtainable by a process according to claim 1 to 4.

6. A composition comprising at least 0.1 wt.% of oil bodies, said oil bodies having a volume weighted mean diameter within the range of 0.1-100 μm and comprising a lipid matrix surrounded by a layer of phospholipids embedded with structural proteins, wherein the lipid matrix contains at least 80 wt.% of glycerides, said glycerides containing at least 3 %, preferably at least 5 % ω-3 C_{20}-C_{24} polyunsaturated fatty acid residues by weight of the total amount of fatty acid residues contained in said glycerides wherein no biomass is attached to the oil bodies or wherein such biomass is attached in an amount of less than 50%, preferably of less than 30% by weight of the oil bodies.

7. Composition according to claim 6, wherein the composition contains at least 1 wt.% of water and the oil bodies are dispersed within the water.

8. Composition according to claim 7, wherein the water contains at least 5 mg per kg of water of a pro-oxidative metal cation selected from iron, copper, zinc and combinations thereof.

9. Composition according to claim 8, wherein the composition contains at least 5 mg/kg of iron.

10. Composition according to any one claim 6 to 9, wherein the oil bodies are derived from a marine micro-organism.

11. Composition according to claim 10 wherein the marine micro-organism is selected from the group consisting of *Crypthecodinium cohnii, Odontella* aurita and *Thraustochytriidae.*

12. Composition according to any one claim 6 to 11, wherein the composition is a food product or a beverage.

13. Composition according to any one claim 6 to 12, wherein the structural protein has a hydrophilic N-terminal portion and/or a hydrophilic C-terminal portion; and a central stretch of at least 25, preferably at least 30 uninterrupted hydrophobic residues.

14. Composition according to claim 13, wherein the structural protein is an oleosin or a caleosin.

15. Use of the oil bodies as defined in claim 5 or the composition as defined in claim 6 in the manufacture of a composition for use in the treatment of prevention of disorders or impaired cognitive function.

16. Use of the oil bodies as defined in claim 5 or the composition as defined in claim 6 in the manufacture of a composition for use in the reduction or prevention of atherosclerosis, cardiovascular disorders or coronary heart disease.

17. Use according to claim 15 in the improvement or treatment of cognitive and mental conditions and disorders as well as the maintenance of normal functions of brain-related systems and processes, preferably ADHD, aging, Alzheimer's disease, Parkinson's disease, multiple sclerosis (MS), dyslexia, depression, learning capabilities, stress, anxiety, concentration and attention.

18. Use according to any one of claims 15-17, wherein the use comprises oral administration of the oil bodies in an amount that is sufficient to deliver at least 10 mg of ω-3 C_{20}-C_{24} polyunsaturated fatty acid residues per administration event.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 07 07 5079

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	16 July 2007	Adechy, Miriam

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X : particularly relevant if taken alone
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A : technological background
O : non-written disclosure
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E : earlier patent document, but published on, or after the filing date
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L : document cited for other reasons

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EPO FORM 1503 03.82 (P04C01)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 07 07 5079

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Case No.: IPR2023-00209
U.S. Patent No. 10,039,306

Espacenet

Bibliographic data: EP2138052 (A1) — 2009-12-30

Gelatinised confectionary, imitation cheese and method for producing same

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Classification: **- international:** ***A23C20/02; A23G3/42; A23G3/48; A23L1/0522; A23L1/09***
- cooperative: **A23C20/02; A23G3/42; A23L1/0522; A23L1/05223; A23L1/095**

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Priority number (s): DE20081030440 20080626

Also published as: EP2138052 (B1) EP2138052 (B8) PL2138052 (T3) ES2534888 (T3) EP2449888 (A1) more

Abstract of EP2138052 (A1)

Gelatinized sweet comprises a liquefied leguminous plant starch or its mixture and at least one modified starch of other origins, where in the mixture, the liquefied leguminous plant starch and the modified starch of other origin is present in the mixture in a mixing proportion of 99:1-1:99 wt.% (on basis of the total mixture). An independent claim is included for preparing the gelatinized sweet, comprising: preparing a mixture containing the liquefied leguminous plant starch or its mixture and the modified starch of other origins, at least one sweetening agent and water; boiling the mixture at 100-150[deg] C to obtain a dried

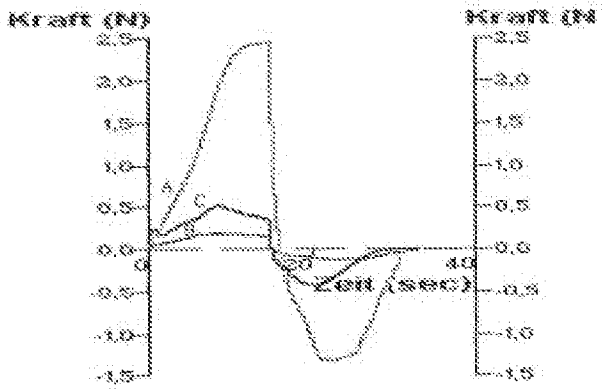


Fig. 1

Case No.: IPR2023-00209
U.S. Patent No. 10,039,306

material; forming the boiled mixture and optionally dividing the mixture; and recovering the obtained gelatinized sweet.

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

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(54) Gelatinierte Süssigkeit, Käseimitat und Herstellungsverfahren

(57) Die vorliegende Erfindung betrifft eine gelatinisierte Süßigkeit, enthaltend eine verflüssigte Hülsenfrucht-Stärke oder eine Mischung aus einer verflüssigten Hülsenfrucht-Stärke und zumindest einer modifizierten Stärke anderen Ursprungs, wobei in der Mischung die verflüssigte Hülsenfrucht-Stärke und die modifizierte Stärke anderen Ursprungs in einem Mischungsverhältnis von 99:1 bis 1:99 Gewichtsprozent (auf Basis der gesamten Mischung) vorliegen.

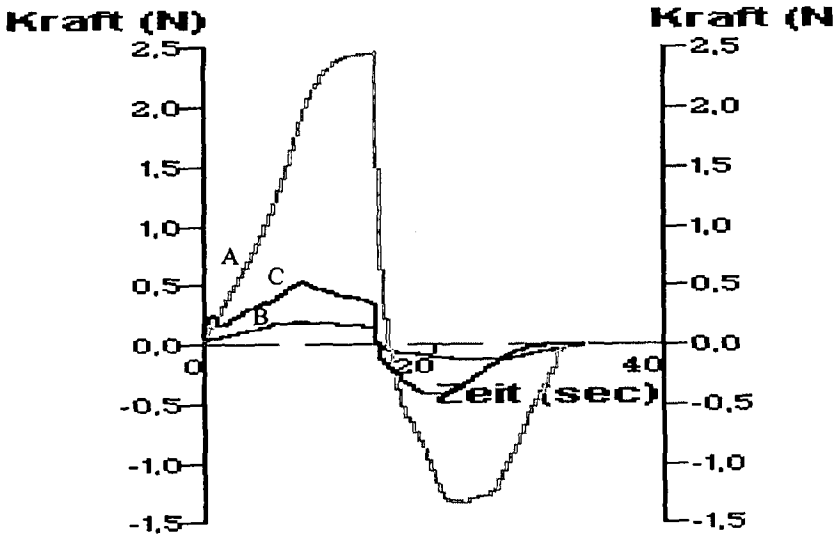


Fig. 1

EP 2 138 052 A1

Beschreibung

[0001] Die vorliegende Erfindung betrifft eine gelatinisierte Süßigkeit, ein Käseimitat sowie ein Herstellungsverfahren derselben

[0002] Aus dem Stand der Technik ist beispielsweise eine auf Stärke basierte, gelatinisierte Süßigkeit aus der EP 1645196 B1 bekannt, die eine verflüssigte und stabilisierte Hülsenfrucht-Stärke mit einem Gehalt an Amylose von 25 bis 40% und ein oder mehrere Süßungsmittel enthält.

[0003] Bei der Herstellung einer solchen Süßigkeit auf Basis von Stärke kann jedoch noch die Gelierzeit verkürzt und die Verarbeitbarkeit insgesamt verbessert werden.

[0004] Es ist somit eine Aufgabe der vorliegenden Erfindung, eine gelatinisierte Süßigkeit bereitzustellen, die die Nachteile des Stands des Technik überwindet, insbesondere eine verkürzte Gelierzeit und eine insgesamt verbesserte Verarbeitbarkeit ermöglicht. Ebenfalls soll ein Herstellungsverfahren für diese Süßigkeit sowie ein Käseimitat bereitgestellt werden.

[0005] Diese Aufgabe wird erfindungsgemäß gelöst durch eine gelatinisierte Süßigkeit, enthaltend eine verflüssigte Hülsenfrucht-Stärke oder eine Mischung aus einer verflüssigten Hülsenfrucht-Stärke und zumindest einer modifizierten Stärke anderen Ursprungs, wobei in der Mischung die verflüssigte Hülsenfrucht-Stärke und die modifizierte Stärke anderen Ursprungs in einem Mischungsverhältnis von 99:1 bis 1:99 Gewichtsprozent (auf Basis der gesamten Mischung) vorliegen.

[0006] Bevorzugt ist die Hülsenfrucht-Stärke eine Erbsenstärke und/oder die modifizierte Stärke anderen Ursprungs eine verflüssigte, stabilisierte und/oder vernetzte Stärke.

[0007] Auch wird vorgeschlagen, dass die Stärke anderen Ursprungs Kartoffelstärke und/oder Tapiokastärke umfasst.

[0008] Ebenfalls kann vorgesehen sein, dass zumindest eine der Stärken getrocknet und/oder vorverkleistert ist.

[0009] Zumindest eine der Stärken kann über Sprühtrocknung hergestellt werden.

[0010] Ferner ist bevorzugt, dass zumindest eine der Stärken dextriniert ist.

[0011] Verflüssigte Stärke kann acetyliert oder hydroxypropyliert sein.

[0012] Auch ist besonders bevorzugt, dass die Süßigkeit 0,5 bis 60 Gew.-%, bevorzugter 0,5 bis 25 Gewichtsprozent an Stärke enthält.

[0013] In einer Ausführungsform ist das Süßungsmittel ein Zucker, der ausgewählt ist aus der Gruppe bestehend aus Mono-, Di- oder Trisacchariden, Oligo- und Polysacchariden, einzeln oder als Mischung.

[0014] Dabei ist bevorzugt, dass das Süßungsmittel ausgewählt ist aus der Gruppe bestehend Maltit, Sorbit, Mannit, Iditol, Erythrit, Xylit, Sorbit-, Maltit-Sirupen, hydrierten Glukose-Sirupen, starken Süßungsmitteln, einzeln oder als Mischung.

[0015] Die Süßigkeit kann ein Gummi- oder Geleeartikel, Lakritz, Dragee oder Kaubonbon sein.

[0016] Erfindungsgemäß ist ein Verfahren zur Herstellung einer gelatinisierten Süßigkeit, wobei es die Schritte umfasst:

- Herstellen einer Zusammenmischung enthaltend verflüssigte Hülsenfrucht-Stärke oder eine Mischung aus einer verflüssigten Hülsenfrucht-Stärke und zumindest einer modifizierten Stärke anderen Ursprungs, wobei in der Mischung die verflüssigte Hülsenfrucht-Stärke und die modifizierte Stärke anderen Ursprungs in einem Mischungsverhältnis von 99:1 1 bis 1:99 Gewichtsprozent (auf Basis der gesamten Mischung) vorliegen, mindestens ein Süßungsmittel und Wasser,

- Kochen der Zusammenmischung bei einer Temperatur zwischen 100 und 150°C, bis ein im wesentlichen eingetrocknetes Material erhalten wird,

- Formen der gekochten Zusammenmischung und gegebenenfalls Aufteilen der Zusammenmischung, und

- Rückgewinnen der so erhaltenen gelatinisierten Süßigkeit.

[0017] Erfindungsgemäß ist schließlich ein Käseimitat, enthaltend verflüssigte Hülsenfrucht-Stärke oder eine Mischung aus einer verflüssigten Hülsenfrucht-Stärke und zumindest einer modifizierten Stärke anderen Ursprungs, wobei in der Mischung die verflüssigten Hülsenfrucht-Stärke und die modifizierte Stärke anderen Ursprungs in einem Mischungsverhältnis von 99:1 bis 1:99 Gewichtsprozent (auf Basis der gesamten Mischung) vorliegen.

[0018] Überraschend wurde gefunden, dass eine erfindungsgemäße Hülsenfrucht-Stärke bzw. eine entsprechende Mischung vorteilhaft bei der Herstellung von Süßigkeiten bzw. Käseimitaten eingesetzt werden kann, da die Verwendung derselben zu einer schnelleren Gelierung und einer besseren Verarbeitbarkeit insgesamt führt. Besonders bevorzugt wird die Hülsenfrucht-Stärke bzw. die Mischung zur Herstellung von Gummi- und Geleeartikeln, Lakritz und Dragees oder Kaubonbons sowie zur Herstellung von Käseimitat eingesetzt. Das Mischungsverhältnis liegt bei 99:1 bis 1:99 für die Mischung aus verflüssigter Hülsenfrucht-Stärke und modifizierter Stärke anderen Ursprungs.

[0019] Wird für die eine verflüssigte Hülsenfrucht-Stärke beispielsweise Erbsenstärke eingesetzt, soll die Stärke anderen Ursprungs dann auch eine andere Hülsenfrucht-Stärke als Erbsenstärke umfassen können.

[0020] Eine vorteilhafte Rezeptur zur Herstellung einer Süßigkeit, wobei alle Prozentangaben in dieser Anmeldung Gewichtsprozentangaben sind, ist wie folgt:

5-30% verflüssigte Erbsenstärke

5-30% verflüssigte und/oder stabilisierte Stärke anderen Ursprungs

10-40% Glukosesirup (42 DE)

10-40% Zucker

10-20% Wasser.

[0021] Im Rahmen der vorliegenden Erfindung ist eine verflüssigte Hülsenfrucht-Stärke eine verflüssigte, jedoch nicht stabilisierte Stärke.

[0022] Eine konkretere Rezeptur zur Herstellung einer erfindungsgemäßen Süßigkeit ist wie folgt:

Verflüssigte Erbsenstärke	20%
Zucker	30%
Glukosesirup (42 DE)	30%
Wasser	20%
Zitronensäure (50%)	12 ml/kg
Aroma	nach Bedarf

[0023] Die erfindungsgemäße Süßigkeit wird hergestellt, indem die einzusetzenden Komponenten zunächst bei einer Temperatur von etwa 60°C miteinander vermischt werden. Die daraus erhaltene Zusammenmischung wird anschließend bei einer Temperatur von etwa 135°C bei einem Druck von 2,5 bar gekocht. Daraufhin kann sie in vorbereitete Formen bei einer Temperatur von etwa 80°C gegossen werden. Hier beträgt die Konzentration der Lösung etwa 70-74° Bx. Nach dem Abfüllen kann die Zusammenmischung bei 20-70°C für eine Dauer von etwa 20-30 Stunden trocknen. Nach dem Trocknen können die Süßigkeiten aus der Form entnommen und abgepackt werden.

[0024] Figur 1 zeigt Texturmessungen unterschiedlicher verflüssigter Stärken mit einem Texture Analyzer, wobei die mit "A" bezeichnete Kurve die Texturmessung für eine erfindungsgemäße eingesetzte Stärke zeigt, Kurve "B" die Messung für eine verflüssigte Weizenstärke und Kurve "C" die Messung für eine modifizierte Kartoffelstärke.

[0025] Die Testbedingungen waren wie folgt: Vorgeschwindigkeit: 1,5 mm/sek; Testgeschwindigkeit: 1,0 mm/sek; Rückgeschwindigkeit: 1,0 mm/sek; Kraft: 100,0 g; Weg: 10,0 mm; Verformung/Strain: 10,0%; Auslösewert: Auto (Kraft); Auslösekraft: 5,0 g; Werkzeug: P/2N-Nadel; Messungen pro Sekunde: 200.

[0026] Zur Messung wurden die Stärkeprodukte in einer 15-%igen Konzentration in Wasser vermischt und aufgekocht bis 95°C, bei 95°C für 10 Minuten gehalten und anschließend auf 25°C abgekühlt. Die Lösung wurde dann bei Raumtemperatur (etwa 22°C) für zwei Stunden stehengelassen und die Textur dann mit einem Texture Analyzer gemessen. Die Stärkelösung gelierte während der Standzeit bei Raumtemperatur.

[0027] Figur 1 zeigt die Ergebnisse der Texturmessung. Für die erfindungsgemäße Süßigkeit wird eine schnellere Gelierung festgestellt, da hier der größte Kraftaufwand für die Texturmessung erforderlich ist.

[0028] Ein erfindungsgemäßes Käseimitat kann mit den folgenden Bestandteilen hergestellt werden:

Verflüssigte und stabilisierte Kartoffelstärke	2,2%
Verflüssigte Erbsenstärke	14,9%
Salz	1,3%
Wasser	59,7%
Zitronensäure	0,2%
Farbstoff	0,1%
Pflanzliches Fett	21,6%
Käse-Aroma	nach Bedarf

[0029] Zunächst wird das Fett in einem Stephan-Mixer erhitzt, bis es geschmolzen ist. Die Trockenstoffe werden mit Wasser vermischt und dann zum Fett zugegeben. Langsam wird bis auf eine Temperatur von oberhalb von 80°C erhitzt und bei dieser Temperatur für 5 Minuten gehalten. Anschließend kann die Masse abgefüllt und dann abgekühlt werden.

[0030] Die in der vorstehenden Beschreibung und in den Ansprüchen offenbarten Merkmale der Erfindung können sowohl einzeln als auch in jeder beliebigen Kombination für die Verwirklichung der Erfindung in ihren verschiedenen Ausführungsformen wesentlich sein.

Patentansprüche

1. Gelatinisierte Süßigkeit, enthaltend eine verflüssigte Hülsenfrucht-Stärke oder eine Mischung aus einer verflüssigten Hülsenfrucht-Stärke und zumindest einer modifizierten Stärke anderen Ursprungs, wobei in der Mischung die verflüssigte Hülsenfrucht-Stärke und die modifizierte Stärke anderen Ursprungs in einem Mischungsverhältnis von 99:1 bis 1:99 Gewichtsprozent (auf Basis der gesamten Mischung) vorliegen.

2. Süßigkeit nach Anspruch 1, wobei die Hülsenfrucht-Stärke eine Erbsenstärke ist und/oder die modifizierte Stärke anderen Ursprungs eine verflüssigte, stabilisierte und/oder vernetzte Stärke ist.

3. Süßigkeit nach Anspruch 1 oder 2, wobei die Stärke anderen Ursprungs Kartoffelstärke und/oder Tapiokastärke umfasst.

4. Süßigkeit nach einem der vorangehenden Ansprüche, wobei zumindest eine der Stärken getrocknet und/oder vorverkleistert ist.

5. Süßigkeit nach einem der vorangehenden Ansprüche, wobei zumindest eine der Stärken dextriniert ist.

6. Süßigkeit nach einem der vorangehenden Ansprüche, wobei verflüssigte Stärke acetyliert oder hydroxypropyliert ist.

7. Süßigkeit nach einem der vorangehenden Ansprüche, wobei sie 0,5 bis 60 Gew.-%, bevorzugt 0,5 bis 25 Gewichtsprozent an Stärke enthält.

8. Süßigkeit nach einem der vorangehenden Ansprüche, wobei das Süßungsmittel ein Zucker ist, der ausgewählt ist aus der Gruppe bestehend aus Mono-, Di- oder Trisacchariden, Oligo- und Polysacchariden, einzeln oder als Mischung.

9. Süßigkeit nach einem der vorangehenden Ansprüche, wobei das Süßungsmittel ausgewählt ist aus der Gruppe bestehend Maltit, Sorbit, Mannit, Iditol, Erythrit, Xylit, Sorbit-, Maltit-Sirupen, hydrierten Glukose-Sirupen, einzeln oder als Mischung.

10. Süßigkeit nach einem der vorangehenden Ansprüche, wobei die Süßigkeit ein Gummi- oder Geleeartikel, Lakritz, Dragee oder Kaubonbon ist.

11. Verfahren zur Herstellung einer gelatinisierten Süßigkeit, wobei es die Schritte umfasst:

    - Herstellen einer Zusammenmischung enthaltend verflüssigte Hülsenfrucht-Stärke oder eine Mischung aus einer verflüssigten Hülsenfrucht-Stärke und zumindest einer modifizierten Stärke anderen Ursprungs, wobei in der Mischung die verflüssigte Hülsenfrucht-Stärke und die modifizierte Stärke anderen Ursprungs in einem Mischungsverhältnis von 99:1 bis 1:99 Gewichtsprozent (auf Basis der gesamten Mischung) vorliegen, mindestens ein Süßungsmittel und Wasser,
    - Kochen der Zusammenmischung bei einer Temperatur zwischen 100 und 150°C, bis ein im wesentlichen eingetrocknetes Material erhalten wird,
    - Formen der gekochten Zusammenmischung und gegebenenfalls Aufteilen der Zusammenmischung, und
    - Rückgewinnen der so erhaltenen gelatinisierten Süßigkeit.

12. Käseimitat, enthaltend verflüssigte Hülsenfrucht-Stärke oder eine Mischung aus einer verflüssigten Hülsenfrucht-Stärke und zumindest einer modifizierten Stärke anderen Ursprungs, wobei in der Mischung die verflüssigte Hülsenfrucht-Stärke und die modifizierte Stärke anderen Ursprungs in einem Mischungsverhältnis von 99:1 bis 1:99 Gewichtsprozent (auf Basis der gesamten Mischung) vorliegen.

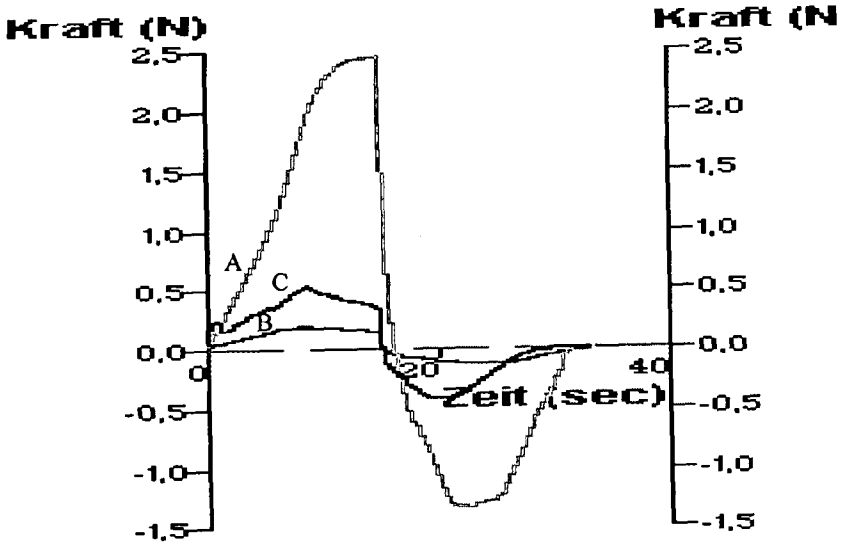


Fig. 1

Europäisches Patentamt
European Patent Office
Office européen des brevets

EUROPÄISCHER RECHERCHENBERICHT

Nummer der Anmeldung
EP 09 00 8258

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Recherchenort	Abschlußdatum der Recherche	Prüfer
Den Haag	14. Oktober 2009	Diller, Reinhard

KATEGORIE DER GENANNTEN DOKUMENTE

X : von besonderer Bedeutung allein betrachtet
Y : von besonderer Bedeutung in Verbindung mit einer anderen Veröffentlichung derselben Kategorie
A : technologischer Hintergrund
O : nichtschriftliche Offenbarung
P : Zwischenliteratur

T : der Erfindung zugrunde liegende Theorien oder Grundsätze
E : älteres Patentdokument, das jedoch erst am oder nach dem Anmeldedatum veröffentlicht worden ist
D : in der Anmeldung angeführtes Dokument
L : aus anderen Gründen angeführtes Dokument

& : Mitglied der gleichen Patentfamilie, übereinstimmendes Dokument

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EPO FORM 1503 03.82 (P04C03)

ANHANG ZUM EUROPÄISCHEN RECHERCHENBERICHT ÜBER DIE EUROPÄISCHE PATENTANMELDUNG NR. EP 09 00 8258

In diesem Anhang sind die Mitglieder der Patentfamilien der im obengenannten europäischen Recherchenbericht angeführten Patentdokumente angegeben.
Die Angaben über die Familienmitglieder entsprechen dem Stand der Datei des Europäischen Patentamts am
Diese Angaben dienen nur zur Unterrichtung und erfolgen ohne Gewähr.

14-10-2009

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Für nähere Einzelheiten zu diesem Anhang : siehe Amtsblatt des Europäischen Patentamts, Nr.12/82

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In diesem Anhang sind die Mitglieder der Patentfamilien der im obengenannten europäischen Recherchenbericht angeführten Patentdokumente angegeben.
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C3H FX
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(58) Field of search
A2B
C3H
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(54) Process for preparing cheese analogs

(57) Synthetic cheese like products are prepared by the steps of forming an aqueous slurry of full-fat oleaginous seed material at a pH below about 3.5, subjecting the aqueous slurry to shear conditions sufficient to disrupt the natural cellular structure of the seed material, adjusting the pH of the slurry to the seed protein's isoelectric point to precipitate protein, separating the entire insoluble portion from the slurry, homogenizing the insoluble portion, adjusting the pH of the homogenized insoluble portion to about 5.3 to 7.0, and blending sufficient flavouring agents therewith to produce a cheese-like flavour. The seed material may be beans, lentils, peanuts, soybeans, rapeseed, safflower, or sunflower. Additional fats may be blended with the product. Gums, colourants, casein, albumin, preservatives, salt and starch may also be present.

GB 2 016 255 A

SPECIFICATION

Process for preparing cheese analogs

BACKGROUND OF THE INVENTION

Dairy-like, in particular cheese-like, analogs from vegetable proteins have been described in U.S. Patents Nos. 4,016,295; 3,891,778; 3,857,977; 3,857,970; 3,809,771; 3,743,516; 3,743,514; and 2,479,040. The extraction of protein from oilseed materials has been discussed in Food Technology 18, (1963); N.Y. Agri. Expt. Sta. Form Res. *28,* 4 (1962) and disclosed in U.S. Patents Nos. 3,968,097; 2,534,227; 2,534,226 and 2,272,562. The effect of pH on the flavor of plant protein extracts has been disclosed in the J. of Food Science *35,* 343 (1970). These references describe various processes and mixtures of vegetable proteins, binders, gums, flavors and colors which have been employed to simulate dairy-like materials. Although the prior art methods result in products having some cheese-like properties, they have generally lacked the desired textural, fatty mouthfeel characteristics of natural cheese. As a result, these products have not been widely accepted by the consumer as true substitutes for natural cheese.

SUMMARY OF THE INVENTION

In accordance with the present invention, a process is provided for preparing improved cheese-like analogs having improved textural and mouthfeel characteristics. The process comprises the steps of forming an aqueous slurry of a full-fat oleaginous seed material at a pH below about 3.5, subjecting the aqueous slurry to shear conditions sufficient to disrupt the natural cellular structure of the seed material, adjusting the pH of the slurry to the oleaginous seed protein's isoelectric point to precipitate the protein, separating the entire insoluble portion from the slurry, homogenizing the insoluble portion, adjusting the pH of the homogenized insoluble portion to about pH 5.3 to 7.0, and blending sufficient flavors therewith to produce a cheese-like flavor.

DESCRIPTION OF THE INVENTION

The full-fat oleaginous seed materials used in the practice of the present invention include legumes, rapeseed, safflower, sunflower and the like. Legumes such as beans, lentils, peanut, soybeans and the like are preferred. The most preferred leguminous seed is soybean.

An aqueous slurry of the full-fat leaginous seed material is formed having a pH below about 3.5. In this pH range, the protein, carbohydrates, flavors and colors from the oleaginous seed material are soluble. The pH is preferably maintained at a value below about 2.2 to produce a fland flavored product. Most preferably, the pH is adjusted to a value between about 1.5 and 2.2. It has been found that a pH much above 3.5 results in the extraction or solubilization of little protein. This acid range is maintained by the addition of any suitable acidic means, such as hydrochloric acid, phosphoric acid, sulfuric acid and the like. Preferably the aqueous slurry contains about 5 to 20 percent oleagenous seed material by weight based upon the total weight of the slurry.

Preferably the aqueous slurry is formed using comminuted, full-fat oleaginous seed material prepared by washing and subdividing the whole full-fat seed material to a fine particle size. Well known means such as grinding, flaking, pulverizing and the like may be employed to comminute the seed material. To avoid the production of off-flavors or odor and to avoid subsequent fat separation, the temperature generated during comminutation should be kept below about 28°C.

The aqueous slurry is then subjected to shear conditions for a period of time sufficient to disrupt the cellular structure of the oleagenous seed material, to reduce the particle size of the oleagenous seed material and to form an uniform, nonparticulate slurry. Suitable means for shearing the slurry include the use of any high speed dispersing, mixing, grinding or homogenizing device. Usually 5 to 10 minutes of shearing is sufficient to disrupt the natural cellular structure. Some particularly troublesome slurries may require longer shearing times up to about 60 minutes.

The pH of the sheared aqueous slurry is then adjusted to the isolectric pH point of the oleagenous protein to precipitate the protein. The isoelectric point, especially for the leguminous seed protein, is between pH 4.4 and 4.8. Suitable basic means, such as sodium hydroxide, potassium hydroxide, trisodium phosphate and the like, may be employed to adjust the pH of the slurry to the desired isolectric point. The insoluble portion of the slurry contains natural fats, fibers, carbohydrates and the like in addition to precipitated protein. In some instances it is desirable to lower the natural fiber and carbohydrate content of the insoluble portion of the aqueous sheared slurry. This can be accomplished by first clarifying the sheared slurry prior to the isoelectric pH adjustment. Suitable means for clarifying the sheared slurry include centrifugation, filtration, decantation and the like.

The resulting insoluble portion of the pH adjusted slurry is then separated from the soluble portion containing soluble carbohydrates, colors and flavors. Suitable separation means include centrifugation, filtration, decantation and the like. It has been found that the texture of the cheese-like end products is influenced by the moisture content of the separated solids. For example, about 45 to 60 percent moisture by weight in the solids results in a product having a hard, dry, sliceable

texture, whereas about 60 to 75 percent moisture by weight in the solids results in product having a semi-soft to soft creamy texture. The moisture content of the insoluble portion can be easily controlled by the removal of moisture by the separation means or by the addition of water thereto.

The separated insoluble portion of the slurry is then homogenized to form a uniform, smooth nonparticulate proteinaceous mixture. Suitable homogenizing means include high speed mixing, grinding, homogenizing device and the like.

The pH of the homogenized slurry is then adjusted to about 5.3 to 7.0. The pH is selected to duplicate the pH of natural cheese in the corresponding cheese-like product to be produced. For example, a cheese-like spread or cream cheese may desirably have a pH of about 5.5 to 6.0 while a semi-soft or hard cheese-like may desirably have a pH of about 5.3 to 6.5. Any suitable basic means as described above may be employed to adjust the pH.

Sufficient flavors may be blended with the homogenized slurry to impart a desired cheese-like flavor. Suitable flavoring means include natural flavors, artificial flavors, reaction products, seasoning and the like which combine to provide a "cheese flavor". The selection of such flavors is well-known to those skilled in the food and flavor art. The amount of flavor added will depend upon the type of flavor and the flavoring means but usually will range between about 0.1 to 30 percent by weight based on the dry weight of the homogenized solids.

The homogenized flavored solids may then be shaped to a desired form. Suitable shaping means include the use of plastic or metal moulds, animal or artificial casings and the like as are well-known for this purpose. To avoid subsequent swelling or foaming, it is desirable to remove air which has been incorporated during processing. Deaeration is simply accomplished by any suitable vacuum means.

In a preferred mode of this process, fat is blended with the homogenized solids to simulate fat-containing natural cheese products, such as cheese spreads, hard cheese and the like. Such fats or oils include saturated or unsaturated fats from animal or plant sources which are either in liquid or solid form at about 20°C. Preferably the fats are the unsaturated types from plant sources. The most preferred fats include soybean oil, corn oil, safflowerseed oil, peanut oil and the like. About 10 to 30 percent fat by weight based on the dry weight of the homogenised solids is blended with the solids. Preferably about 20 to 25 percent fat by weight based on the dry weight of the solids is blended therewith. To insure uniform blending of the fats with the homogenised solids, it is preferable to maintain the temperature during blending at about 60°C.

In order to prepare cheese-like products having semi-soft to hard cheese-like textures, the shaped mixture is heated for a sufficient period of time to set the protein in a stable, firm physical form. The temperature and heating period selected have a direct influence on the firmness and the moisture content (dryness) of the finished product. Preferably the shaped mixture is heated to an internal temperature of about 70°C to 100°C. The use of temperatures from about 82°C to 95°C for about 30 to 60 minutes result in hard textured products. Lower temperatures of from about 70° to 80°C for similar heating periods result in a softer textured product. A suitable temperature and heating period thus can be selected to produce a product having the desired texture and moisture content.

Other ingredients may be added to the homogenized solids to improve the color, meltability, mouthfeel, taste or texture of the cheese-like product. Such ingredients include vegetable gums, artificial and natural colorants, casein, albumin, preservatives, salts, starch and the like which are well-known for these purposes.

The following examples further illustrate the present invention and are not intended to limit the breadth described above.

EXAMPLE 1

This example illustrates the preparation from soybeans of a cheese analog with a hard cheese-like texture.

Whole, full-fat soybeans (22.7 kilograms) were ground in a Fitz mill to pass through a 0.125 inch screen opening and then added to an aqueous mixture at pH 1.7 containing 272.7 kilograms water, 7.2 kilograms phosphoric acid (85%, food grade), 114 grams sodium sulfite and 28 grams of an antifoaming agent (this mixture contains about 7.6 weight percent soybeans based on the total weight of the slurry), After mixing, the pH of the slurry rose to pH 2.2. The aqueous slurry was then passed through an in-line shearing device (Tekmar Dispenser type OR-3-616, Tekmar Co. Cincinnati, Ohio) having three (coarse, medium and fine) grinding heads and into a holding tank. The pH of the sheared slurry was then adjusted to pH 4.6 by the addition of 10 weight percent aqueous sodium hydroxide to precipitate the protein. The insoluble portion containing precipitated protein and other insoluble constituents was then separated from the soluble portion of the slurry by centrifugation. The separated solids contained about 83 weight percent water. The separated solids were then passed through an homogenizer (Manton Gaulin type 15M8TRA) to produce a smooth textured uniform mixture. The pH of the homogenized mixture was adjusted to pH 5.8 by the addition of 10

weight percent sodium hydroxide.

Four hundred grams of the resulting mixture were heated to about 71°C and blended with 95 ml water, 80 grams melted Crisco (hydrogenated soybean oil, 75°C), 2.5 grams of hydrated calcium carageenan in 60 ml water and 92 grams of cheese flavors. The mixture was blended for 30 minutes to a uniform smooth consistency. The mixture was then deaerated *in vacuo*, moulded and heated in a steam bath for 45 minutes (the internal temperature reached 90°C during the last 10 minutes of heating) to form a stable shaped form. After cooling, the shaped product was removed from the mould. The resulting cheese analog had the texture, mouthfeel and flavor of a hard cheese similar to cheddar cheese.

EXAMPLE 2

This example illustrates the preparation from peanuts of a cheese analog having a semi-hard cheese-like texture.

Raw, whole, blanched, full-fat peanuts (500 grams) were added to 3000 ml of water which had been previously adjusted to pH 1.6 by the addition of 54 ml of phosphoric acid (85%, food grade). This mixture contained about 14.3 weight percent peanuts based on the total weight of the slurry. The aqueous mixture was then subjected to shear conditions using a submerged grinder (Tekmar type SD-45) for 10 minutes to comminute the peanuts and form a uniform non-particulate slurry. After grinding, the pH of the slurry was 2.1. The pH of the sheared slurry was then adjusted to pH 4.6 by the addition of 10% aqueous sodium hydroxide to precipitate the protein and other insoluble constituents. The insoluble portion was filtered from the slurry and collected onto layers of cheese cloth without squeezing. Two hundred ml of water were added to the separated mixture to adjust the solids content to about 28 weight percent. The resulting solids were passed through a hand homogenizer to produce a uniform, smooth-textured mixture. The pH of the homogenized mixture was adjusted to pH 5.8 by the addition of 10 weight percent aqueous sodium hydroxide. The moist solids were then heated to 50°C and 35 grams of melted Crisco were added with constant stirring. After about 5 minutes, 1.0 gram of hydrated calcium carageenan was added and followed by the addition of 30 grams of cheese flavor. After the ingredients were uniformly blended, the mixture was placed in a metal mould and heated in a steam bath for 45 minutes. The internal temperature of the mixture reached about 90°C during heating. The mixture was cooled and the shaped product removed from the mould. The resulting cheese analog had the texture, mouthfeel and flavor of a semi-hard cheese similar to Colby cheese.

EXAMPLE 3

This example illustrates the preparation from soybeans of cheese analogs having textures similar to various types of cheese.

Whole, full-fat soybeans (22.7 kilograms) were added to an aqueous mixture at about pH 1.65 containing 272.7 kilograms water, 6.36 kg of phosphoric acid (38%, food grade) and 114 grams of sodium sulfite (this mixture contains about 7.6 weight percent soybeans based on the total weight of the mixture). The entire mixture was then passed through an in-line shearing device (Tekmar Disperser type DR-3-616) to reduce the size of the soybeans to a fine particle size. The insoluble portion of the sheared mixture containing insoluble cellular material (fiber, carbohydrates) was separated by centrifugation from the soluble portion containing protein, fats and other soluble constituents. The pH of the soluble portion was adjusted to pH 4.6 by the addition of aqueous sodium hydroxide (50%, 757 ml) to precipitate protein and other constituents insoluble at this pH. The precipitated insoluble solids were removed from the mixture by centrifugation and washed in the centrifuge with 272.7 kg of water. The separated solids, about 22 kilograms, had a moisture content of about 68 weight percent. The solids were analyzed and found to contain the following on a weight percent basis:

Protein	57.8
Fat	34.6
Carbohydrates (by difference)	7.6

A. Preparation of a cheese analog similar to cream cheese

Four hundred grams of the separated solids prepared above were vigorously agitated in a laboratory blender to a smooth uniform consistency. The pH of the agitated solids was adjusted to pH 6.0 by the addition of 10 weight percent aqueous sodium hydroxide. The mixture was then moulded and refrigerated to serving temperature. The product was creamy, smooth and bland in taste similar in texture to cream cheese.

B. Preparation of a cheese analog similar to flavored cream cheese spread

Four hundred grams of the separated solids prepared above were vigorously agitated in a laboratory blender to a smooth uniform consistency. The pH of the agitated solids was adjusted to pH 5.5 using 10 weight percent aqueous sodium hydroxide. Eighty grams of artificial cheese flavors was uniformly blended with the mixture. The product was moulded and refrigerated to serving temperature. The cheese analog was creamy smooth with a good cheese flavor similar to flavored cream cheese spreads.

C. Preparation of a cheese analog similar to processed cheese spread

Four hundred grams of the separated solids prepared above were vigorously agitated in a laboratory blender to a smooth uniform consistency. The pH of the agitated solids was adjusted to pH 6.1 by the addition of 20 weight percent aqueous trisodium phosphate. The mixture was heated to about 60°C. Eighty grams of melted Crisco fat and 1 gram of Keltrol F (xanthan gum) were blended with the mixture. Then 80 grams of natural and artificial cheese flavors and about 180 ml of water were blended with the mixture. The mixture was heated to 60°C and mixed for 16 minutes. The product was moulded and refrigerated to serving temperature. The cheese analog had the texture similar to processed cheese spreads.

D. Preparation of a cheese analog similar to commercial soft cheese.

One thousand grams of the separated solids prepared above were vigorously agitated in a laboratory blender to a smooth uniform consistency. The pH of the agitated mixture was adjusted to pH 5.3 by the addition of 20 weight percent trisodium phosphate. To the mixture there was then added 12 grams of sodium chloride and 94.8 grams of artificial and natural cheese flavors. The mixture was blended for about 30 minutes. The product was deaerated *in vacuo,* moulded and heated in a steam bath for 45 minutes. The internal temperature of the analog reached about 90°C during heating. The product was refrigerated to serving temperature. The cheese analog had a medium firm texture which did not cut clean with a knife and was similar to a commercial soft cheese.

CLAIMS

1. A process for preparing cheese-like analogs, which comprises

(a) forming an aqueous slurry containing a full-fat oleaginous seed material having a pH below about 3.5;

(b) subjecting the aqueous slurry to shear conditions sufficient to disrupt the natural cellular structure and reduce the particle size of the oleaginous seed material;

(c) adjusting the pH of the resulting sheared aqueous slurry to the oleaginous seed protein's isoelectric point to precipitate the protein;

(d) separating the entire insoluble portion of the slurry from the soluble portion;

(e) homogenizing the insoluble portion of the slurry to a uniform nonparticulate consistency;

(f) adjusting the pH of the homogenized slurry to about pH 5.3 to 7.0; and

(g) blending sufficient flavouring agents with the homogenized slurry to produce a cheese-like flavour.

2. A process according to Claim 1 wherein the aqueous slurry contains 5 to 20 percent oleaginous seed material by weight based upon the total weight of the slurry.

3. A process according to Claim 1 or 2 wherein the oleaginous seed material is leguminous seed.

4. A process according to Claim 3 wherein the leguminous seed is soybean.

5. A process according to any preceding claim, wherein the full-fat oleaginous seed material in the slurry is comminuted.

6. A process according to any preceding claim wherein 0.1 to 30 percent of flavouring agents, by weight based on the dry weight of homogenized solids, are blended with the homogenized solids.

7. A process according to any claim preceding wherein fat is blended with the homogenized slurry.

8. A process according to Claim 7 wherein 10 to 30 percent fat, by weight based on the dry weight of homogenized solids, is blended with the homogenized slurry.

9. A process according to claim 7 or 8 wherein the fat-containing blend is heated to about 60°C.

10. A process according to any preceding claim further comprising shaping the homogenized flavoured solids to a desired form.

11. A process according to Claim 10 wherein the shaped solids are heated to set the protein in a stable physical form.

12. A process according to Claim 10, wherein fat is blended with the homogenized slurry, the fat-containing blend is heated to about 60°C and the shaped solids are heated to an internal temperature of about 70°C to 100°C.

13. A process according to Claim 12 wherein the shaped solids are heated to an internal temperature of about 70°C to 80°C for a sufficient period of time to form a semi-soft cheese-like analog.

14. A process according to Claim 12 wherein the shaped solids are heated to an internal temperature of about 82° to 95°C for a sufficient period of time to form a hard cheese-like analog.

15. A process according to any preceding claim, wherein the sheared aqueous slurry is clarified before its pH is adjusted to the isoelectric point of the protein.

16. A process according to Claim 1, substantially as described in Example 1, 2, 3A, 3B, 3C, or 3D.

17. A synthetic cheese-like foodstuff produced by a process claimed in any preceding claim.

18. A cream cheese analog produced by a process claimed in Claim 1.

19. A cheese spread analog produced by a process claimed in Claim 9.

20. A semi-soft cheese analog produced by a process claimed in Claim 13.

21. A hard cheese analog produced by a process claimed in Claim 14.

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Bibliography

DWPI Title

Cheese substitute prodn. from soybean milk comprises fermenting soybean milk with starting soln. of lactic acid bacteria, cutting, shaping, adding mould starting soln. and fermenting

English Title

METHOD OF PRODUCING CHEESEE LIKE FOOD FROM SOYBEAN MELK

Assignee/Applicant

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JP197672936A / 1976-06-21 / JP

Abstract

DWPI Abstract

(JP52156962A_)

Novelty

Soybean milk is fermented with the starting soln. of lactic acid bacteria, cutting the obtd. curd into pieces, heat-standing, moulding, pressing them, adding the starting soln. of mould, fermenting and ripening.The starter soln. is of a thermo-resistible lactic acid bacteria e.g., Streptococcus lactis, Streptococcus thermophilus, etc. The mould starter soln. is Penicillium caseicolum etc. Curd is fermented with the mould until the water soluble N content is increased to 15-70% and is ripened in absence of oxygen.Cheese substitute is equal to cheese produced from milk in tissue, colour, appearance, taste and flavour.

Classes/Indexing

IPC

IPC Code(1-7) **A23C 19/02** A23L 1/20

(7)

Current IPC	Invention	Version	Additional	Version
Full	A23C 20/02 A23C 19/02 A23C 20/00 A23G 3/00 A23G 3/34 A23L 1/20	20060101 20060101 20060101 20060101 20060101 20060101	-	-
Main Group	-	-	-	-
Subclass	-	-	-	-

JP FI Codes

A23C 20/00 ; A23C 20/02 ; A23G 3/00

JP F Terms

4B001: AC08; AC30; AC31; AC46; BC08; BC14; EC01

4B014: GG06; GG15

DWPI Class

D13

DWPI Manual Codes

Expand DWPI Manual Codes

DWPI Chemistry Resource Numbers

Expand DWPI Chemistry Resource Numbers

DWPI Registry Numbers

Expand DWPI Registry Numbers

Plasdoc Punch Codes

Expand Plasdoc Punch Codes

Plasdoc Key Serial Codes

Expand Plasdoc Key Serial Codes

Enhanced Polymer Indexing

Expand Enhanced Polymer Indexing

Chemical Fragment Codes

Expand Chemical Fragment Codes

Legal Status

INPADOC Legal Status

Get Family Legal Status

Family

Family

Expand INPADOC Family (2)

Expand DWPI Family (1); Countries (1)

Claims

No Claims exist for this Record

Description

Drawing Description

Expand Drawing Description

Description

Expand Description

Citations

Citation *Record level*

Expand Citing Patents (4)

Cited Patents (0)

Cited Non-patents (0)

DPCI Citation *Patent family level*

Expand DPCI Citing Patents (2)

DPCI Cited Patents (0)

DPCI Cited Non-patents (0)

Expand DPCI Citation Counts

DPCI Field of Search

-

DPCI Citing Patents Country

JP; WO

DPCI Cited Patents Country

-

Other

DWPI Title Terms

CHEESE SUBSTITUTE PRODUCE SOY MILK COMPRISE FERMENTATION START SOLUTION LACTIC ACID BACTERIA CUT SHAPE ADD MOULD

Custom Fields

Test

-

Relevance

-

Product Name

-

Docket number

-

Notes

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（全 4 頁）

⑭大豆乳を利用したチーズ様食品の製造法

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㉒出　　願　昭51(1976)6月21日

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明　　細　　書

1. 発明の名称　　大豆乳を利用したチーズ様食品の製造法

2. 特許請求の範囲

(1) 大豆乳に乳酸菌スターターを添加し醗酵せしめ、当該醗酵により生成したカードを切断、加熱放従、搾詰め、圧搾し、次いでカビスターターを接種し醗酵、熟成せしめることを特徴とするチーズ様食品の製造法。

(2) カビスターター接種後、全窒素中の水溶性窒素が15～70%になる迄醗酵熟成せしめ、次いで酸素との接触を遮断しつつ熟成せしめる特許請求の範囲第1項記載のチーズ様食品の製造法。

(3) 乳酸菌スターターが、耐塩性乳酸菌スターターである特許請求の範囲第1項または第2項記載のチーズ様食品の製造法。

3. 発明の詳細な説明

本発明は大豆乳を利用したチーズ様食品の製造法、更に詳しくは大豆乳を原料とした風味良好なしかも組織、色調、外観上も牛乳からのチーズに匹敵するカビチーズ様食品の製造法に関する。

大豆蛋白を利用して種々の食品が盛んに開発され、食肉類似大豆蛋白等にあつては実用化される迄になつている。また大豆蛋白よりチーズ様食品の開発も研究がなされているが、牛乳からのチーズに匹敵する風味良好な食用可能な製品の開発には未だ成功していないのが現状である。

従来、豆乳から風味良好なチーズ様食品が取得できない主原因は豆乳からのカードと牛乳からのカードではその熟成時に於ける組成、組織が極めて異つているからである。即ち、豆乳からのカードの含水量が牛乳からのものに比し極めて高く、またカードの組成が豆乳からのものは牛乳からのものに比し熟成に不適当なためにカードの熟成がゆるやかに円滑に進行せず風味の醸成が不可能になるためとされて来た。従つて、豆乳から可能な

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絞り含水量の低いカードを製造することが不可欠であつて、そのために牛乳の場合に凝固剤としてレンネットを使用すると同様に豆乳に外部より積極的に凝固剤、主としてカルシウム塩を添加しカードを調製し、それを原料として用いて来たのである。また豆乳よりのチーズ様食品の製造法に関する改良研究も大いになされているが、その大部分はいかに含水量の低いカードを製造するかに係るものである。

本発明者らは、上記一般的技術通念にとらわれることなく、全く新しい観点に立ち風味良好なチーズ様食品を製造する技術を確立すべく鋭意研究を行つた結果、風味醸成のためには含水量が主要因となるのではなく、むしろカルシウム、デルタダルコノラクトン等の添加凝固剤が熟成を阻害すること、乳酸菌のみを使用するのではなくカビによる熟成、熟成が風味、色調、外観ともに良好なチーズ様食品の製造には不可欠であること、更に牛乳の場合と異り耐熱性乳酸菌を使用することが組織、風味向上に好ましいことを知見し、これら知見に

基づき本発明を完成するに至つた。

即ち、本発明は、

豆乳に乳酸菌スターターを添加し醗酵せしめ、当該醗酵により生成したカードを切断、加温放置、凝結め、圧搾し、次いでカビスターターを接種し熟成せしめることによるチーズ様食品の製法である。

本発明の第1段階は丸大豆全脂大豆粉体或いは脱脂大豆等からの豆乳の調製にある。豆乳の調製は従来法により行えばよいが、殺菌は牛乳の場合の低温殺菌（約65℃、30分間）と異り高温殺菌を行うことが重要である。即ち、牛乳の場合には共存する微生物群がチーズ熟成に関与し好結果を与えるので低温殺菌が行われるが、豆乳を原料とする場合には共存する雑菌はかえつて製品の風味を害う原因となるので高温殺菌を採用することとなる。丸大豆からの具体的な豆乳の調製法を示すと、まず丸大豆に水を加え、約倍量の吸水ある迄浸漬する。この浸漬は通常12～16時間にて完了する。次いで浸漬丸大豆を磨砕、90～100

℃にて約10分間加熱し殺菌と同時に蛋白質を抽出し、遠心分離、濾過等の固液分離手段にて豆乳を分離することにより調製される。全脂大豆粉末、或いは脱脂大豆からの豆乳の調製も上記とほゞ同様に行えばよいが、脱脂大豆を使用する場合には外部より油脂を添加乳化せしめるのが得られる製品品質上好ましい。豆乳調製用原料としては上述の如くいかなるものでもよいが、より高品質の製品を得るには丸大豆を原料とするのが最も好ましい。

如くして得られた豆乳に耐熱性乳酸菌スターターを添加し醗酵を行うのが本発明方法の第二段階である。醗酵温度は30～40℃にて行えばよく、牛乳からのチーズ、特にカビチーズ製造の場合と同様に、ストレプトコッカス・ラクチス、ストレプトコッカス・クリモリス等の通常の乳酸菌を用いても行いえるが、耐熱性乳酸菌の使用がより好適である。これはカビスターター接種迄の各工程にて乳酸菌が死滅或いは極度に減少すると風味の著干劣る製品が得られる為であつて、カビスター

ターによる醗酵熟成時にあつても乳酸菌が生存、生育することにより熟成中のpHの変化を緩慢にし、しかもカビが増殖であるカードのpHを生育環境に適するよう調整するために生成する代謝物を徐々に蓄積させ、風味醸成を促すので好適である。このような耐熱性乳酸菌は40℃以上にあつても生育するものであつて、例えばストレプトコッカス、サーモファイラス、ラクトバチルス、デルブリュッキイ等が使用される。またこの耐熱性乳酸菌と通常の乳酸菌を併用することにより乳酸醗酵時間の短縮をはかれるが、製品の品質としては耐熱性菌のみにても充分である。乳酸菌スターターの添加は、予め豆乳または脱脂乳等に培養した乳酸菌スターターを豆乳に対し3%程度加えることにより行われる。一方、原料豆乳としては本発明方法の第1段階に従い調製した豆乳のみにても充分牛乳からのチーズに近似する製品を得ることができるが、脱脂乳等の動物性乳の添加も差しつかえない。

本乳酸醗酵はカード生成迄行えばよいのである

Motif Exhibit 1148, Page 457 of 3341

が、通常乳酸濃度0.29%以上にてカードが生成するのでその時点で次工程へ進むことになる。本発明方法は前述したとおり、カード生成を外部より凝固剤を添加することなく乳酸醗酵にて生成する乳酸のみにて行う点が一つの特徴である。即ち、凝固剤、例えばカルシウム塩、デルタグルコノラクトン等を添加した場合には熟成が円滑に進行せず熟成率（水溶性窒素／全窒素×100）が30%と低く、しかも風味も組織も良好なものでないが、本発明方法にあつては熟成が円滑に進行すると同時になめらかな組織が得られ初めて食用に供せられるチーズ様食品が製造可能となるのである。

次いで本発明方法の第3段階として生成カードを切断、加温放置、整詰め、圧搾しカビスターター接種用原料を調製する。生成カードの処理温度は牛乳を原料とする場合より高温を必要とし、40～50℃に20～30分要して加温し、切断、次いで同温度にて短時間、通常10～20分間放置することが好ましい。この温度に於加温することにより組織の滑らかな良質カードとすることがで

きると同時に次工程の熟成をより円滑に行うことができる。整詰め、圧搾は常法により行えばよく、特に強度の圧搾は必ずしも必要としない。前述したとおり大豆蛋白は高保水性を有し強度の圧搾を行つても含水量の低下にはそれ程効果なく、通常のプレス圧にて一晩圧搾するだけで充分である。かくして得られたカビスターター接種用カードは含水量約70%と通常の牛乳からのカードの約50%に比較し極めて高いが、得られた製品は牛乳からのチーズと同等の品質を有するのである。

第3段階で得た整詰めカードよりカードを取り出し、カビスターターを接種し、必要に応じ食塩を乾塩法等により添加後醗酵熟成するのが、最終の第4工程である。本発明者の知見によれば、豆乳を原料とする場合には乳酸菌のみにて長期熟成させても熟成率は極めて低く食用に供し得るチーズ様食品の取得は困難であるが、カビチーズスターターによる熟成により目的がはじめて達成されるので、本工程は不可欠である。カビスターターの接種量は、パン培地に培養した後凍結乾燥した種

品にてカードに対し約0.3%程度にて充分であり、食塩量は約2%程度である。カビスターターとしてはペニシリウム、カゼイコラム、ペニシリウム、カマンベルテイ等牛乳からのカビチーズの製造に用いられるカビが使用可能であるが、特に白かび即ちペニシリウム　カゼイコラムが風味の点で特に好適である。

熟成は15～16℃にて極めて円滑に進行し2～3週間目にかけて急速に進行する。従つて2～3週間の熟成にて風味良好な大豆臭のないしかも色調、外観、組織の極めて優れたチーズ様食品が得られる。

上記のとおり熟成が2～3週間目にて急速に進行し、場合によつては以後の保存中に過熟気味になり、カビスターターとの接触面が黒ずむことが認められたので、この点に関し更に研究した。その結果、15～16℃にて約1～2週間醗酵熟成を行つた後、即ち熟成率が15～70%になる迄醗酵熟成を行つた後、液体部分を除去しまたはせずして後酸菜との接触をパラフインコーティング

等の手段にて遮断し、以後熟成、好ましくは低温例えば5℃にて熟成せしめることにより上記の欠点が克服できるとともに保存性が向上することを見い出した。熟成率が15%以下では後段の熟成を行つても風味良好な食用に供せるチーズ様食品になる迄熟成せず、また70%以上ではカビスターターとの接触部が過熟し黒ずむ危険性があるので第一段の醗酵熟成は熟成率が15～70%、より好適には20～50%にとどめることが肝要である。本2段熟成によれば後段の熟成後3～4週間経過しても熟成率は80～90%であり、過熟による異風味の生成、カビスターターとの接触面の黒ずみ等は認められず、風味、外観ならびに保存性の一層の向上に有効であることが確認された。

以下、実施例により本発明をより詳しく説明する。

実施例　1

北海道産白目大豆1500gに水を加え、16時間浸漬し、次いで10倍量の水の存在下に磨砕

し、９５℃にて１０分間加熱殺菌、蛋白抽出を行い遠心分離し固形分濃度5.7％の豆乳１２４００ｇを得た。

上記豆乳を４０℃に冷却後市販のストレプトコッカス・ラクチスおよびストレプトサーモフイラスを脱脂粉乳中にて培養した培養液の等量混合液を豆乳に対し３％添加し４０℃にて醗酵せしめた。

カードの生成が認められた時点で、生成カードを２０～３０分間かけ５０℃に加温し、次いで常法に従い切断、同５０℃にて１５分間放置した後内径７３㎜のステンレス鋼製フープに型詰めした。その後、５００ｇ重量のプレスをのせ一晩圧搾した。

型より取り出したカード（ｐＨ 4.７５）に予めパン培地に培養し、凍結乾燥した後粉末化したペニシリウム・カゼイコラム（ＡＴＣＣ ６９８６）のカビスターターをカードに対して0.３％接種、また食塩を２％乾塩法にて混和添加した。次いで１５～１６℃にて３週間熟成したところ、風味良好にして組織、外観、色調に優れたチーズ様

比較のために、上記製造法にあつて乳酸菌スターター添加２時間後５０℃にて豆乳に対し0.５％の割合にて塩化カルシウムを添加し、以後同様にしてチーズ様食品を製造した。

本比較品はカビ菌体との接触面のみ褐変して黒色を呈しており、アンモニア臭が認められたが内部迄熟成は進行していなかつた。また熟成率および遊離アミノ酸量は夫々３０％および3.２㎎／ｇ試料を極めて劣つていた。

実施例　2

実施例1.と同様にして得たカビスターター添加カードを１５～１６℃にて８日間(A)および２週間(B)緩慢熟成後（熟成率(A)２５％、(B)６６％）、カビ菌体部分を除去、パラフインコーテイングし、５℃にて熟成した。

低温熟成を(A)については５週間、(B)については３週間行つたところ、熟成率は夫々、７４％、６３％で過熟による異風味は認められず、風味、組織、色調ならびに外観とも実施例１のものより

食品が得られた。

風味の一つの指標となる熟成率および遊離アミノ酸量は夫々８８％および１１.５㎎／ｇ試料であつた。また遊離脂肪酸量は2.０１㎎／ｇ試料で、これは牛乳から製造した硬質チーズに匹敵していた。

本品を男子１０名、女子４０名に試食させ、５段階の評価を求めたところ、次の結果を得た。この結果から明らかなとおり、本発明に係るチーズ様食品は充分食用に供せられる優れたものであることが判る。

<結　果>

	男性(人)	女性(人)	回答率(%)		回答率(%)
			男性	女性	総合
好ましい	3	5	3０	12.5	16.0
少し好ましい	6	25	6０	62.5	62.0
中位	1	4	１０	１０.１	１０.０
少し好ましくない	0	5	0	12.5	１０.０
好ましくない	0	1	0	2.5	2.0

優れていた。

特許出願人　松　岡　博　厚

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(54) Title: USES OF OIL BODIES

(57) Abstract

The present invention provides novel emulsion formulations which comprise oil bodies. The invention also provides a method for preparing the emulsions and the use of the emulsions in various domestic and industrial compositions. The emulsions are especially suited for the preparation of food products, personal care products, pharmaceutical products and industrial products.

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Title: USES OF OIL BODIES

FIELD OF THE INVENTION

The present invention provides novel emulsions which comprise oil bodies. The invention also provides a method for preparing the emulsions and the use of the emulsions in various domestic and industrial compositions.

BACKGROUND OF THE INVENTION

Emulsions are mixtures which are prepared from two mutually insoluble components. It is possible to generate mixtures of homogenous macroscopic appearance from these components through proper selection and manipulation of mixing conditions. The most common type of emulsions are those in which an aqueous component and a lipophilic component are employed and which in the art are frequently referred to as oil-in-water and water-in-oil emulsions. In oil-in-water emulsions the lipophilic phase is dispersed in the aqueous phase, while in water-in-oil emulsions the aqueous phase is dispersed in the lipophilic phase. Commonly known domestic examples of emulsion-based formulations include mayonnaise, margarine, ice cream, cosmetics and paint. Emulsion systems are also extensively applied in industries such as the pharmaceutical and the agrochemical industries, where it is often desirable to formulate active ingredients in emulsions.

Generally emulsions are prepared in the presence of a multiplicity of other substances in order to achieve a desirable balance of emulsification, viscosity, stability and appearance. For example, the formulation of emulsions usually requires at least one, and frequently a combination of several, emulsifying agents. These agents facilitate the dispersal of one immiscable phase into the other and assist in stabilizing the emulsion. Emulsifiers comprise a wide variety of synthetic and natural components. For example, monoglycerides and chemical derivatives thereof, are widely used as emulsifiers in food applications such as margarines and baked products. An example of a natural emulsifier is lecithin, a phospholipid present in egg yolk, which is commonly used in the preparation of mayonnaise. It is also possible to entrap active ingredients in emulsions. This is especially desirable in compositions comprising active agents which are difficult to dissolve in aqueous solutions, such as certain vitamins and nucleotides. Active ingredients are also frequently formulated as emulsions in order to enhance their stability. One example of an emulsion system comprising a pharmaceutical agent is documented in US patent 5,602,183 which discloses a wound healing composition containing an anti-inflammatory agent. The foregoing exemplifies only a few of the myriad of components which are included in formulations of emulsions known in the art. A comprehensive overview of emulsifying agents and their applications may be found in Becher, P. Encyclopedia of Emulsion Technology, Dekker Ed., 1983.

In the seeds of oilseed crops, which include economically important crops, such as soybean, rapeseed, sunflower and palm, the water insoluble oil fraction is stored in

discrete subcellular structures variously known in the art as oil bodies, oleosomes, lipid bodies or spherosomes (Huang 1992, Ann. Rev. Plant Mol. Biol. 43: 177-200). Besides a mixture of oils (triacylglycerides), which chemically are defined as glycerol esters of fatty acids, oil bodies comprise phospholipids and a number of associated proteins, collectively termed oil body proteins. From a structural point of view, oil bodies are considered to be a triacylglyceride matrix encapsulated by a monolayer of phospholipids in which oil body proteins are embedded (Huang, 1992, Ann. Rev. Plant Mol. Biol. 43: 177-200). The seed oil present in the oil body fraction of plant species is a mixture of various triacylglycerides, of which the exact composition depends on the plant species from which the oil is derived. It has become possible through a combination of classical breeding and genetic engineering techniques, to manipulate the oil profile of seeds and expand on the naturally available repertoire of plant oil compositions. For an overview of the ongoing efforts in his area, see Designer Oil Crops/Breeding, Processing and Biotechnology, D. J. Murphy Ed., 1994, VCH Verlagsgesellschaft, Weinheim, Germany.

Plant seed oils are used in a variety of industrial applications, notably in the food, detergent and cosmetics industries. In order to obtain the plant oils used in these applications, seeds are crushed or pressed and subsequently refined using processes such as organic extraction, degumming, neutralization, bleaching and filtering. Aqueous extraction of plant oil seeds has also been documented (for example, Embong and Jelen, 1977, Can. Inst. Food Sci. Technol. J. 10: 239 - 243). Since the objective of the processes taught by the prior art is to obtain pure oil, oil bodies in the course of these production processes lose their structural integrity. Thus, the prior art emulsions formulated from plant oils do not generally comprise intact oil bodies.

Although there are many applications where mineral oil based products dominate the market, in other applications, oils derived from plant sources and fossil sources are in direct competition. Lauric oils, for example, which are widely used in the manufacture of detergents, are obtained from mineral oil as well as from coconut oil and more recently from genetically engineered rapeseed (Knauf, V. C., 1994, Fat. Sci. Techn. 96: 408). However, there is currently an increasing demand for biodegradable sources of raw materials. The plant oil body based emulsions of the present invention offer an advantage over similar mineral oil based formulations, in that the oil fraction is derived from a renewable and environmentally friendly source.

United States patents 5,683,710 and 5,613,583 disclose emulsions comprising lipid vesicles from oleaginous plants. The emulsions disclosed in these patents are prepared from relatively crude seed extracts and comprise numerous seed components including glycosylated and non-glycosylated proteins. It is a disadvantage of the emulsions to which these patents relate that they comprise contaminating seed components imparting a variety of undesirable properties, which may include allergenicity and undesirable

odour, flavour, colour and organoleptic characteristics, to the emulsions. Due to the presence of seed contaminants, the preparations of lipid vesicles disclosed in these patents have limited applications.

SUMMARY OF THE INVENTION

The present invention relates to novel emulsion formulations which contain oil bodies. The emulsion formulations of the subject invention are obtainable in non-toxic and food grade forms. In addition, the emulsion formulations are advantageously prepared from an oil body preparation which is creamy in texture and thus may be readily applied in a variety of domestic and industrial applications. The present inventors have found that the oil body fraction of living cells is useful in the formulation of a variety of novel emulsion-based food, cosmetic, pharmaceutical and industrial products. Broadly stated, the present invention provides an emulsion formulation comprising washed oil bodies derived from a cell.

The invention also provides methods for preparing the emulsion formulations and the use of the emulsion formulations in various domestic and industrial compositions.

Accordingly, the present invention provides a method for preparing emulsion formulations comprising: 1) obtaining oil bodies from a cell; 2) washing the oil bodies; and 3) formulating the washed oil bodies into an emulsion.

In a preferred embodiment of the invention, the washed oil body preparation is obtained from plant seeds, including seeds obtainable from rapeseed, soybean, maize and sunflower. Accordingly, the invention provides a method for preparing the emulsion formulations from plant seeds comprising:

(a) grinding plant seeds;

(b) removing solids from the ground seeds;

(c) separating the oil body phase from the aqueous phase;

(d) washing the oil body phase to yield a washed oil body preparation;

(e) formulating the washed oil body preparation into an emulsion.

In a preferred embodiment of the invention, a liquid phase is added to the seeds prior to or following grinding of the seeds.

In a further preferred embodiment of the invention, formulating the emulsion (e) comprises adding a liquid phase to the washed oil body preparation.

The emulsions of the present invention can be used in a wide range of applications including in the preparation of food and feed products, pharmaceutical products, personal care products, and industrial products. The emulsion formulation of the present invention is especially suited for the preparation of food-grade products as it is non-toxic, creamy in texture and biodegradable.

Additional objects, advantages and features of the present invention will become apparent after consideration of the accompanying drawings and the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a Coomassie blue stained gel of a washed oil body preparation from white mustard, rapeseed (*Brassica napus*), soybean, peanut, squash, flax, sunflower, safflower and maize.

Figure 2A-C are Coomassie blue stained gels showing the protein profiles of various seed fractions obtained from *Brassica napus* (Canola) (A), sunflower (B), and maize (C). The gels show the following fractions (1) total seed protein (TSP), (2) decanted liquid phase (DL), (3) unwashed oil bodies (LP1), (4) three washes with water (LP4), (5) four washes with water and one wash with 100 mM Na_2CO_3 (Washed).

DETAILED DESCRIPTION OF THE INVENTION

As hereinbefore mentioned, the present invention relates to emulsion formulations comprising oil bodies derived from a cell. In one embodiment, the present invention provides an emulsion formulation comprising washed oil bodies.

In another embodiment, the present invention provides a method for preparing an emulsion formulation comprising: 1) obtaining oil bodies from a cell; 2) washing the oil bodies; and 3) formulating the washed oil bodies into an emulsion.

The cell can be any cell that contains oil bodies (or oil-body like structures) including plant cells, animal cells, fungal cells and bacterial cells. In a preferred embodiment of the invention the oil bodies are obtained from a plant cell. The oil bodies may be obtained from a plant cell by rupturing the plant cell membrane and cell wall using any method which releases the cells constituents without substantially compromising the structural integrity of the oil bodies. More preferably, the oil bodies are obtained from plant seeds. Accordingly, the present invention further provides a method for preparing an emulsion formulation comprising:

(1) obtaining oil bodies from plant seeds by a method that comprises:
  (a) grinding plant seeds;
  (b) removing solids from the ground seeds; and
  (c) separating the oil body phase from the aqueous phase;
(2) washing the oil body phase to yield a washed oil body preparation; and
(3) formulating the washed oil body preparation into an emulsion.

In a preferred embodiment of the invention, a liquid phase is added to the seeds prior to or following grinding of the seeds.

In a further preferred embodiment of the invention, formulating the emulsion (e) comprises adding a liquid phase to the washed oil body preparation.

The term "grinding" as used herein means milling, crushing, chopping or granulating the seeds and these terms may be used interchangeably throughout this application. In the process, the seed cells are broken open.

The term "solids" as used herein means any material that is not soluble in the aqueous phase or in the oil body phase, such as seed hulls.

The term "washing the oil bodies" as used herein means any process that removes cellular contaminants from the oil body phase, in particular any contaminant which imparts undesirable properties to the emulsion formulation, such as allergenic properties, undesirable colour, odour, flavour or organoleptic characteristics or any other undesirable property. Examples of methods of washing include gravitation based separation methods such as centrifugation and size exclusion based separation techniques such as membrane ultrafiltration and crossflow microfiltration. Washing methods and conditions are selected in accordance with the desired purity of the oil body preparation.

The term "washed oil body preparation" as used herein means a preparation of oil bodies from which a significant amount of cellular material has been removed including contaminants which impart undesirable properties to the emulsion formulation, such as allergenic properties, undesirable colour, odour, taste or organoleptic characteristics or any other undesirable property. Preferably, the washed oil body preparation contains less than 10% of other seed proteins.

By "formulating the oil bodies into an emulsion", it is meant that the washed oil body preparation is mixed or homogenized, if necessary, until an emulsion is formed. In a preferred embodiment, an additional ingredient is added, such as a liquid phase, and the washed oil body preparation and the liquid phase are mixed until a homogenous mixture is attained.

The washed oil body preparations are particularly suitable for the formulation of emulsions due to advantageous properties outlined below.

PROPERTIES OF THE OIL BODIES

The emulsion formulations of the present invention comprise intact washed oil bodies of approximately uniform size, shape and density. When viewed under the electron microscope, oil bodies are found to be more or less spherically shaped structures (see: Example Murphy, D. J. and Cummins I., 1989, Phytochemistry, 28: 2063-2069; Jacks, T. J. et al., 1990, JAOCS, 67: 353-361). Typical sizes of oil bodies vary between 0.4 µm for and 1.5 µm (Murphy, D. J. and Cummins I., 1989, Phytochemistry, 28: 2063-2069). When analyzed using a Malvern Size Analyzer, it was found that oil bodies in a washed oil body preparation isolated from rapeseed were symmetrically and unimodally distributed around 1 µm. Using a Malvern Size Analyzer a washed oil body preparation could be clearly distinguished from commercially obtainable oil-in-water emulsions including soymilk, mayonnaise (Kraft Real Mayonnaise) and two coconut milk preparations (Tosca, Aroy-D).

The exact size and density of the oil bodies depends at least in part on the precise protein/phospholipid/triacylglyceride composition which is present. Preparing washed oil bodies according to the present invention does not result in a substantive alteration in the shape of the oil bodies in comparison with those present in whole seed when viewed under the electron microscope.

Upon breaking open a cell containing oil bodies, the oil body fraction may be rapidly and simply separated from aqueous solutions since in aqueous solutions the oil body fraction will float upon application of centrifugal force. In solutions, where the density of the oil body fraction is greater than that of the solvent, such as 95% ethanol, the oil bodies will sediment under the same conditions. The oil body fraction may also be separated from the aqueous fraction through size-exclusion based separation techniques, such as membrane filtration, which may be advantageous in that more uniformly sized oil bodies may be acquired.

The oil bodies present in the washed oil body preparations of the present invention are resistant to exposure to strong acids and bases, including prolonged exposure to acidic conditions at least as low as pH 2 and alkaline conditions at least as high as pH 10. When exposed to pH 12, a slight loss of oil was observed, indicating a loss of integrity of the oil body structure. In addition, extraction with various organic solutions, including methanol, ethanol, hexane, isopropyl alcohol and ethyl acetate, does not or only slightly compromise the integrity of the oil bodies present in the washed oil body preparation. The oil bodies present in the washed oil body preparation were also found to withstand mixing with the anionic detergent, sodium dodecyl sulfate (SDS), the cationic, detergent hexadecyl trimethyl bromide and Tween-80, a non-ionic detergent. Boiling of the washed oil body preparation in the presence of SDS was found to result at least partly in disintegration of the oil body structure. The oil bodies present in the washed oil body preparation are stable when maintained for 2 hours up to at least 100°C. A slow freeze and thaw of washed oil body preparations resulted in a change in their physical appearance characterized by the formation of clumps as opposed to a homogeneous emulsion. Oil body clumping following a freeze-thaw could also be prevented to a large degree by either a) flash freezing in liquid nitrogen instead of slow freezing at -20°C or b) adding glycerol in excess of 5% (v/v) to the oil body preparation prior to freezing. The resistance to relatively harsh chemical and physical conditions, is a unique characteristic of the oil bodies present in the washed oil body preparation of the subject invention.

The present invention provides emulsion formulations comprising oil bodies from which a significant amount of seed contaminants have been removed. These contaminants include proteins, volatiles and other compounds which may impart undesirable colour, odour, flavour, organoleptic characteristics or other undesirable characteristics. A number of seed proteins have been reported to cause allergenic reactions.

For example, Ogawa et al. (1993, Biosci. Biotechnol. Biochem., 57:1030-1033) report allergenicity of the soybean glycoprotein P34 (alternatively referred to as Gly m Bd 30K). Allergenic reactions against rapeseed, wheat and barley seed proteins have also been reported (Armentia et al., 1993., Clin. Exp. Allergy 23: 410-415; Monsalve et al., 1993, Clin. Exp. Allergy 27: 833 - 841). Hence removal of contaminating seed proteins is advantageous. Washing conditions may be selected such that a substantially pure oil body preparation is obtained. In that case, only the oil body proteins are substantially present in the preparation.

For many applications, it is also considered desirable that a purer better defined oil body preparation is obtained, as this allows more control over the formulation process of the final emulsion. In order for the washed oil body preparation to be included in a diverse set of emulsions it is desirable that volatiles are kept to a minimum and the colour is preferably light or white. Washing of the oil body preparation results in a lighter coloured preparation. In addition, a substantial amount of volatiles is removed. Also removed by washing are compounds which promote the growth of microorganisms as it was observed that a washed oil body preparation had a longer shelf life than an unwashed preparation. Other compounds which are removed by washing include anti-nutritional glucosinilates and/or breakdown products thereof and fibrous material. When heat treated to 60°C or 80°C, it was observed that larger quantities of water remained absorbed by the washed oil body preparation when compared with an unwashed preparation. Upon cooling down to room temperature and centrifugation, it was observed that the washed oil body preparation remained stable, while phase separation occurred in the unwashed preparation. Given the enhanced stability of washed oil bodies, they are preferred where the formulation process involves the application of heat. When heated to 40°C, the washed oil body preparation was able to absorb a larger quantity of exogenously added water without resulting in phase separation. Thus in the formulation of aqueous emulsions, washed oil bodies are preferred. The capacity to absorb exogenously added oils was also compared between a preparation of washed oil bodies and an unwashed preparation. Larger amounts of exogenous oil could be added to the washed oil body preparation before an unstable emulsion was formed. This is advantageous in formulations where exogenous oils or waxes are added in the formulation process such as where lubricants or cosmetics are prepared. When viscosity was compared between a washed oil body preparation and an unwashed preparation it was found that the washed preparation was more viscous. A more viscous preparation of oil bodies is desirable as this eliminates the need for the addition of thickening agents in the formulation process.

Thus the washed oil body preparation provided here is superior to an unwashed preparation in many respects. The washed oil body preparation of the present invention is a better defined preparation with a longer shelf life and more preferable

colour, odour and viscosity characteristics. The washed oil body preparation also has superior water and oil absorption characteristics. Finally due to the removal of a significant amount of seed proteins, allergenic reactions are less likely to occur. These characteristics allow the use of the washed oil body preparation in the formulation of a variety of domestic and industrial emulsions.

The above observations were made using washed and unwashed oil body preparations obtained from rapeseed and prepared as detailed in Example 2 of the present application. It is believed that resistance to relatively harsh chemical and physical conditions will be a characteristic of the oil bodies present in the washed oil preparation of the subject invention regardless of the source of the oil bodies. However it is likely that one or more of the hereinbefore documented properties for rapeseed oil bodies will vary somewhat depending on the cells from which the washed oil bodies preparation is obtained. Nevertheless it is to be clearly understood that the subject invention is drawn to an oil body preparation which may be obtained from any cell comprising oil bodies.

In one embodiment of the present invention, the oil bodies are obtained from plant seeds. The presence of intact oil bodies in the emulsion and the described characteristics of these oil bodies clearly distinguish the subject emulsion formulation from other materials which may be prepared from plant seeds.

SOURCES AND PREPARATION OF THE OIL BODIES

The washed oil body preparation of the subject may be obtained from any cell containing oil bodies or oil body-like organelles. This includes animal cells, plant cells, fungal cells, yeast cells (Leber, R. et al., 1994, Yeast 10: 1421-1428), bacterial cells (Pieper-Fürst et al., 1994, J. Bacterol. 176: 4328 - 4337) and algae cells (Rossler, P.G. , 1988, J. Physiol. (London) 24: 394-400). In preferred embodiments of the invention the oil bodies are obtained from a plant cell which includes cells from pollens, spores, seed and vegetative plant organs in which oil bodies or oil body-like organelles are present (Huang, 1992, Ann. Rev. Plant Physiol. 43: 177-200). More preferably, the washed oil body preparation of the subject invention is obtained from a plant seed and most preferably from the group of plant species comprising: rapeseed (*Brassica* spp.), soybean (*Glycine max*), sunflower (*Helianthus annuus*), oil palm (*Elaeis guineeis*), cottonseed (*Gossypium* spp.), groundnut (*Arachis hypogaea*), coconut (*Cocus nucifera*), castor (*Ricinus communis*), safflower (*Carthamus tinctorius*), mustard (*Brassica* spp. and *Sinapis alba*), coriander (*Coriandrum sativum*), squash (*Cucurbita maxima*), linseed/flax (*Linum usitatissimum*), Brazil nut (*Bertholletia excelsa*), jojoba (*Simmondsia chinensis*) and maize (*Zea mays*). Plants are grown and allowed to set seed using agricultural cultivation practises well known to a person skilled in the art. After harvesting the seed and if desired removal of material such as stones or seed hulls (dehulling), by for example sieving or rinsing, and optionally drying of the seed, the seeds are subsequently processed by mechanical pressing, grinding or crushing. In a

preferred embodiment, a liquid phase is added prior to grinding of the seeds. This is known as wet milling. Preferably the liquid is water although organic solvents such as ethanol may also be used. Wet milling in oil extraction processes has been reported for seeds from a variety of plant species including: mustard (Aguilar et al 1990, Journal of Texture studies 22:59-84), soybean (US Patent 3,971,856; Carter et al., 1974, J. Am. Oil Chem. Soc. 51:137-141), peanut (US Patent 4,025,658; US Patent 4,362,759), cottonseed (Lawhon et al., 1977, J. Am. Oil, Chem. Soc. 63:533-534) and coconut (Kumar et al., 1995, INFORM 6 (11):1217-1240). It may also be advantageous to imbibe the seeds for a time period from about fifteen minutes to a about two days in a liquid phase prior grinding. Imbibing may soften the cell walls and facilitate the grinding process. Imbibition for longer time periods may mimic the germination process and result in certain advantageous alterations in the composition of the seed constituents. In another embodiment, the liquid phase is added after the seeds are ground. This is known as dry milling. Preferably the added liquid phase is water.

The seeds are preferably ground using a colloid mill, such as the MZ130 (Fryma Inc.). Besides colloid mills, other milling and grinding equipment capable of processing industrial scale quantities of seed may also be employed in the here described invention including: flaking rolls, disk mills, colloid mills, pin mills, orbital mills, IKA mills and industrial scale homogenizers. The selection of the mill may depend on the seed throughput requirements as well as on the source of the seed which is employed. It is of critical importance that seed oil bodies remain intact during the grinding process. Therefore, any operating conditions commonly employed in oil seed processing, which tend to disrupt oil bodies are unsuitable for use in the process of the subject invention. Milling temperatures are preferably between 10°C and 90°C and more preferably between 26°C and 30°C, while the pH is preferably maintained between 2.0 and 10.

Solid contaminants, such as seed hulls, fibrous material, undissolved carbohydrates and proteins and other insoluble contaminants, are removed from the crushed seed fraction. Separation of solid contaminants, may be accomplished using a decantation centrifuge, such as a HASCO 200 2-phase decantation centrifuge or a NX310B (Alpha Laval). Depending on the seed throughput requirements, the capacity of the decantation centrifuge may be varied by using other models of decantation centrifuges, such as 3-phase decanters. Operating conditions vary depending on the particular centrifuge which is employed and must be adjusted so that insoluble contaminating materials sediment and remain sedimented upon decantation. A partial separation of the oil body phase and liquid phase may be observed under these conditions.

Following the removal of insoluble contaminants, the oil body phase is separated from the aqueous phase. In a preferred embodiment of the invention a tubular bowl centrifuge is employed. In other embodiments, hydrocyclones, disc stack centrifuges, or settling of phases under natural gravitation or any other gravity based separation method

may be employed. It is also possible to separate the oil body fraction from the aqueous phase employing size exclusion methods, such as membrane ultrafiltration and crossflow microfiltration. In preferred embodiments the tubular bowl centrifuge is a Sharples model AS-16 (Alpha Laval) or a AS-46 Sharples (Alpha Laval). A critical parameter is the size of the ring dam used to operate the centrifuge. Ring dams are removable rings with a central circular opening varying, in the case of the AS-16, from 28 to 36 mm and regulate the separation of the aqueous phase from the oil body phase thus governing the purity of the oil body fraction which is obtained. In preferred embodiments, a ring dam size of 29 or 30 mm is employed when using the AS-16. The exact ring dam size employed depends on the type of oil seed which is used as well as on the desired final consistency of the oil body preparation. The efficiency of separation is further affected by the flow rate. Where the AS-16 is used flow rates are typically between 750-1000 ml/min (ring dam size 29) or between 400-600 ml/min (ring dam size 30) and temperatures are preferably maintained between 26°C and 30°C. Depending on the model centrifuge used, flow rates and ring dam sizes must be adjusted so that an optimal separation of the oil body fraction from the aqueous phase is achieved. These adjustments will be readily apparent to a skilled artisan.

Separation of solids and separation of the aqueous phase from the oil body fraction may also be carried out concomitantly using a gravity based separation method such as 3-phase tubular bowl centrifuge or a decanter or a hydrocyclone or a size exclusion based separation method.

The compositions obtained at this stage in the process, generally are relatively crude and comprise numerous seed proteins, which includes glycosylated and non-glycosylated proteins and other contaminants such as starch or glucosinilates or breakdown products thereof. The present invention comprises the removal of a significant amount of seed contaminants. To accomplish removal of contaminating seed material, the oil body preparation obtained upon separation from the aqueous phase is washed at least once by resuspending the oil body fraction and centrifuging the resuspended fraction. This process yields what for the purpose of this application is referred to as a washed oil body preparation. The number of washes will generally depend on the desired purity of the oil body fraction. Depending on the washing conditions which are employed, an essentially pure oil body preparation may be obtained. In such a preparation the only proteins present would be oil body proteins. In order to wash the oil body fraction, tubular bowl centrifuges or other centrifuges such hydrocyclones or disc stack centrifuges may be used. Washing of oil bodies may be performed using water, buffer systems, for example, sodium chloride in concentrations between 0.01 M and at least 2 M, 0.1 M sodium carbonate at high pH (11-12), low salt buffer, such as 50 mM Tris-HCl pH 7.5, organic solvents, detergents or any other liquid phase. In preferred embodiments the washes are performed at high pH (11-12). The

liquid phase used for washing as well as the washing conditions, such as the pH and temperature, may be varied depending on the type of seed which is used. Washing at a number of different pH's between pH 2 and pH 11-12 may be beneficial as this will allow the step-wise removal of contaminants, in particular proteins. Washing conditions are selected such that the washing step results in the removal of a significant amount of contaminants without compromising the structural integrity of the oil bodies. In embodiments where more than one washing step is carried out, washing conditions may vary for different washing steps. SDS gel electrophoresis or other analytical techniques may conveniently be used to monitor the removal of seed proteins and other contaminants upon washing of the oil bodies. It is not necessary to remove all of the aqueous phase between washing steps and the final washed oil body preparation may be suspended in water, a buffer system, for example, 50 mM Tris-HCl pH 7.5, or any other liquid phase and if so desired the pH may be adjusted to any pH between pH 2 and pH 10.

The process to manufacture the washed oil body preparation may be performed in batch operations or in a continuous flow process. Particularly when tubular bowl centrifuges are used, a system of pumps operating between steps (a) and (b), (b) and (c), and (c) and (d) a continuous flow throughout the processing system is generated. In a preferred embodiment, the pumps are 1 inch M2 Wilden air operated double diaphragm pumps. In other embodiments, pumps, such as hydraulic or peristaltic pumps may be employed. In order to maintain a supply of homogenous consistency to the decantation centrifuge and to the tubular bowl centrifuge, homogenizers, such as an IKA homogenizer may be added between the separation steps. In-line homogenizers may also be added in between various centrifuges or size exclusion based separation equipment employed to wash the oil body preparations. Ring dam sizes, buffer compositions, temperature and pH may differ in each washing step from the ring dam size employed in the first separation step.

In embodiments of the invention where the oil bodies are isolated from softer tissues, for example the mesocarp tissue of olives, the techniques applied to break open the cell may vary somewhat from those used to break harder seeds. For example, pressure-based techniques may be preferred over crushing techniques. The methodology to isolate oil bodies on a small scale has been reported for isolation of oil bodies from mesocarp tissues in olive (*Olea europaea*) and avocado (*Persea americana*) (Ross et al., Plant Science, 1993, 93: 203-210) and from microspore-derived embryos of rapeseed (*Brassica napus*) (Holbrook et al., Plant Physiol., 1991, 97: 1051-1058).

In embodiments of the invention where oil bodies are obtained from non-plant cells, the washed oil body preparation is isolated following similar procedures as outlined above. The methodology to isolate oil bodies from yeast has been documented (Ting et al., 1997, Journal Biol. Chem. 272:3699-3706).

The chemical and physical properties of the oil fraction may be varied in at least two ways. Firstly, different plant species contain oil bodies with different oil compositions. For example, coconut is rich in lauric oils (C_{12}), while erucic acid oils (C_{22}) are abundantly present in some *Brassica* spp. Secondly, the relative amounts of oils may be modified within a particular plant species by applying breeding and genetic engineering techniques known to the skilled artisan. Both of these techniques aim at altering the relative activities of enzymes controlling the metabolic pathways involved in oil synthesis. Through the application of these techniques, seeds with a sophisticated set of different oils are obtainable. For example, breeding efforts have resulted in the development of a rapeseed with a low erucic acid content (Canola) (Bestor, T. H., 1994, Dev. Genet. 15: 458) and plant lines with oils with alterations in the position and number of double bonds, variation in fatty acid chain length and the introduction of desirable functional groups have been generated through genetic engineering (Töpfer et al., 1995, Science, 268: 681-685). Using similar approaches a person skilled in the art will be able to further expand on the presently available sources of oil bodies. Variant oil compositions will result in variant physical and chemical properties of the oil bodies. Thus by selecting oilseeds or mixtures thereof from different species or plant lines as a source for oil bodies, a broad repertoire of emulsions with different textures and viscosities may be acquired.

FORMULATING THE EMULSION

The washed oil body preparation may be formulated into an emulsion using techniques known in the art. Preferably, at least one additional ingredient is added to the washed oil body preparation. The additional ingredient may be added as a solution, suspension, a gel or solid and quantities of the additional ingredient will depend on the formulation. The additional ingredient may upon formulation become associated with the oil bodies, remain suspended in solution, or form a suspension in which the oil bodies are dispersed. The ingredient may also penetrate the phospholipid monolayer surrounding the oil body or the triacylglyceride matrix. Ingredients which may penetrate the oil body include oils, waxes and the colorant Nile Red. In a preferred embodiment, the additional ingredient is a liquid phase. In a further preferred embodiment the liquid phase is water. Water may be added either directly or through moisture associated with another ingredient. The final amount of water is not critical, as long as upon mixing of the ingredients, a stable emulsion is formed. Generally, the compositions will contain at least 1% of water and up to 99% water. Usually mixing will be required to provide an adequate emulsion and it may be necessary to apply heat or pressure.

In another preferred embodiment the additional ingredient is an oil or a wax. Oils or waxes may partition to the triacyl glyceride matrix of the oil bodies and in this manner lipid soluble ingredients, such as lipid soluble vitamins may be delivered to

the oil body matrix. Where oils or waxes comprise the added ingredient, the oil bodies may remain suspended in the lipophilic phase or double emulsions may be formed.

The final compositions may be in solid or in liquid form or of any other desired viscosity. The emulsion may be thickened using gelling agents such as cellulose and derivatives, Carbopol and derivatives, carob, carregeenans and derivatives, xanthane gum, sclerane gum, long chain alkanolamides, and bentone and derivatives, typically present in concentrations less than 2% by weight.

The emulsion may further comprise surfactants to wet, foam, penetrate, emulsify, solubilize and or disperse a selected material. For example anionic surfactants such as sodium coconut monoglyceride sulphonate, cationic surfactants, such as lauryl trimethyl ammonium chloride, cetyl pyridinium chloride and trimethylammonium bromide, nonionic surfactants including pluronics, and polyethylene oxide condensates of alkyl phenols, and zwitterionic surfactants such as derivatives of aliphatic quaternary ammonium, phosmomium and sulphonium compounds may all be added as required.

Chelating agents, capable of binding metal ions, such as tartaric acid, EDTA, citric acid, alkali metal citrates, pyrophosphate salts or anionic polymeric polycarboxylates may be also included in the emulsion formulation as desired.

Generally, the emulsion formulations will be treated such that contamination by bacteria, fungi, mycoplasmas, viruses and the like or undesired chemical reactions, such as oxidative reactions are prevented. In preferred embodiments this is accomplished by the addition of preservatives, for example sodium metabisulfite or other chemical additives or by irradiation, for example by ionizing radiation such as cobalt-60 or cesium-137 irradiation or by ultraviolet irradiation.

In addition, active agents may be added to the washed oil body preparation. For example cosmetic compositions may be formulated as stable suspensions using the present emulsion formulation and vitamins and moisturizing agents may be included in skin creams. One particularly advantageous way in which an active ingredient may be included in emulsions of the subject invention, is through construction of oleosin gene fusions as detailed in WO 96/21029. Briefly stated, WO 96/21029 discloses a method of producing proteins and peptides as fusion proteins of oleosins. These fusion proteins are created by genetically linking the gene encoding oleosin to a gene encoding a peptide or protein of interest. Expression of the fusion gene, in for example an oilseed plant, results in synthesis of a fusion protein which is then targeted to the oil body. Isolation of the oil body fraction results in recovery of the fusion protein. In principle any desired protein or peptide may be produced using this technology. For example, it is envisaged that polar fish antifreeze peptides (Davies, P.L. et al. 1990, FASEB J. 4: 2460-2468) are produced as oleosin fusion proteins. The washed oil body preparation may then be employed to prepare ice creams, milkshakes or other frozen foodgrade materials with improved freezing

properties by inhibiting or preventing ice crystal formation. In another example, a therapeutic protein may be produced as an oleosin fusion. The oil bodies may then be used to formulate a desirable suspension which may be for oral consumption, or for topical skin application. This embodiment of the present invention is further exemplified in example 11 of the present invention where a fish food is prepared which comprises oil bodies comprising an oleosin - carp growth hormone fusion

An emulsion with film forming properties may also be formulated. Such an emulsion when it is applied to a surface and dried forms a coating. An example of an emulsion where a coated oil body film is applied is in fish food, where oil bodies may be applied to the fish food to enhance the dietary value. A film forming emulsion is particularly useful in embodiments of the present invention where controlled release of an active ingredient is desirable such as in delivery of pharmaceuticals or volatiles such as fragrances. The release time of the active agent from a film of emulsion, which occurs during drying, depends, among other factors, on the thickness of the film. When a thicker coating is applied a longer drying time will result in a slower release of the active agent. In variant contemplated formulations, release of the agent occurs only when the film is dry. Other factors, such as the composition of the emulsion and the type and concentration of the active ingredient also determine the characteristics of release. For example, cosolvents, such as ethanol, may be included in the formulation and influence the release time. Release of an active ingredient is also desirable in food applications, where a flavorant entrapped in an emulsion is released during consumption. The release of the flavorant, depending on the exact formulation of the emulsion, may elicit a sudden intense sensation or a more subtle blend of flavours and essences.

The emulsion formulation may also be used in sprays and aerosols. Preferably small sized oil bodies, for example 1 μm or less in diameter such as those found in *B. napus*, are used for this purpose. Volatiles, such as alcohol and fragrances may be included in these sprays. Emulsions of this type may also be sprayed onto the surface of dried food preparations such as potato chips and dried soup. The emulsion might include a flavorant and add preservative value or assist in maintaining the appropriate moisture levels of the food.

USES OF THE EMULSION FORMULATION

The subject invention is directed toward the production of emulsions that are useful in industrial and domestic compositions. It is noted that the emulsions may be applied in compositions which vary widely in physical properties and use. Thus specific embodiments include applications such as food and feed products, pharmaceutical products, personal care products and industrial products.

Food and feed uses include non-dairy substitutes, such as non-dairy cheese or yoghurt, margarines, mayonnaises, vinaigrettes, icings, ice creams, salad dressings,

synthetic mustards, candy, chewing gum, pudding, baking products, condiments, juice clouding agents, baby formula, flavour carriers, texturing agents (shortening), pet food, fish food and livestock feed. Personal care products applications include soaps, cosmetics, skin creams, facial creams, tooth paste, lipstick, perfumes, make-up, foundation, blusher, mascara, eyeshadow, sunscreen lotions, hair conditioner, and hair colouring. Pharmaceutical products which may be formulated using the washed oil body preparation of the subject invention include therapeutic agents, diagnostic agents and delivery agents. As a therapeutic or diagnostic agent, the emulsion will additionally contain an active ingredient. The active ingredient can be anything that one wishes to deliver to a host. In one embodiment, the active ingredient may be a protein or peptide that has therapeutic or diagnostic value. Such peptides include antigens (for vaccine formulations), antibodies, cytokines, blood clotting factors and growth hormones. Industrial uses for the emulsions of the present invention include paints, coatings, lubricants, films, gels, drilling fluids, paper sizing, latex, building and road construction material, inks, dyes, waxes, polishes and agrochemical formulations. In preferred embodiments, the subject invention is directed to compositions which may be ingested by animals and humans. Since, these compositions may be ingested they must be of foodgrade quality. The particular product and the particular form in which the emulsion is applied, however is not of critical importance and may be as desired. It is to be clearly understood that the emulsion formulated with the washed oil body preparation may be applied in any domestic or industrial product.

The stability of the present emulsion formulation at low pH may be exploited in formulations of acid emulsions. For example, the emulsion formulation may be used in the preparation of a mayonnaise-like foodproduct, which besides the washed oil body preparation comprises a vegetable oil, mustard, vinegar and egg yolk, if desired. Pourable emulsions, such as salad dressings may be prepared by increasing the relative amount of vinegar and/or by the addition of water.

An example of an application where heat may be applied without apparent deleterious effects, is in the preparation of a savory sauce such as a bechamel sauce or in sweet sauces such as chocolate sauces. In these applications, the washed oil body preparation is employed as a frying substitute. To prepare a bechamel sauce, to 1 part of the heated washed oil body preparation, 1 part (w/w) of flour is added and stirred until a thick suspension is formed. At moderate heat milk is gradually added until a sauce with a desired viscosity is obtained.

The emulsion formulation may also be used as a butter substitute. In this application, small amounts of water are added to the washed oil body preparation, for example, less than 10% until a desired viscosity is obtained. Natural butter flavours and thickeners may be added as desired. The butter substitute may be used on sweet corn, bread, in cake mixes or bread making. Salt, which contributes flavour and acts as a preservative

may be added typically to a level of about 2.5% (wt/vol). Colour agents, for example, extracts of annatto seed or carotene may be added to deepen the colour as desired. An advantage of this application is that the oil body based butter does not comprise hydrogenated fatty acids, which are used in the formulations of margarines and the like to achieve a desirable consistency, but are also with associated with cardiovascular diseases.

Shortenings may be prepared to various degrees of stiffness, from a foam to a pourable shortening. In this application, air is beaten into the emulsion formulation and the emulsion formulation can be considered to be dispersed into the continuous phase, air. Shortenings may be applied to mixes where creaming and fluffing are desired. These mixes include icings, synthetic creams, ice creams and cake batter.

An imitation fruit juice may be prepared from artificial or natural flavours and nutrients. Such imitation juices do not have the correct appearance and due to transparency appear to be weak or diluted. By adding a small amount, for example 0.1 to 1% (v/v) of the washed oil body preparation or an emulsion thereof clouding may occur to give the juice a rich appearance. Thus the present oil body preparation may be used as a clouding agent.

In another application involving juices, the washed oil body preparation or an emulsion thereof may be added to juices with settleable solids, such as tomato juice. Adding a small amount of the washed oil body preparation, for example 0.1 to 1% (v/v), may decrease the rate of settling of the solids in the juice and assist in maintaining the rich appearance.

Topical applications of the washed oil body preparation of the present invention are also envisaged. In this embodiment the emulsion is formulated as a dermatologically acceptable emulsion, which may for example be employed to moisturize facial and/or body skin, including nails and lips or may have properties to combat ageing of the skin, acne, pigmentation, hair loss, or promote hair removal or facilitate wound healing and/or restructuring of the skin tissue. The washed oil body preparation represents preferably 1-99% by weight of the final composition.

The cosmetic compositions of the present invention may comprise additional hydrocarbon compounds such as plant, animal, mineral or synthetic oils or waxes or mixes thereof. They comprise paraffin, petrolatum, perhydrosqualene, arara oil, almond oil, calphyllum oil, avocado oil, sesame oil, castor oil, jojoba oil, olive oil, or cereal germ oil. Esters may be included such as esters of lanolic acid, oleic acid, lauric acid, stearic acid, myristic acid. It is also possible to include alcohols for example, oleoyl alcohol, linoleyl alcohol or linolenyl alcohol, isostearyl alcohol or octyl dodecanol, alcohol or polyalcohol. Further hydrocarbons which may be included are octanoates, decanoates, ricinoleates, caprylic/capric triglycerides or C_{10} to C_{22} fatty acid triglycerides. Addition of these agents may result in the formation of double emulsions.

Hydrogenated oils, which are solid at 25°C, such as hydrogenated castor oil, palm oil or coconut oil, or hydrogenated tallow; mono- di- tri- or sucroglycerides; lanolins; and fatty acids which are solid at 25°C may also be included in the cosmetic formulations of the present invention. Among the waxes which may be included are animal waxes such as beeswax; plant waxes such as carnauba wax, candelilla wax, ouricurry wax, Japan wax or waxes from cork fibres or sugar cane; mineral waxes, for example paraffin wax, lignite wax, microcrystalline waxes or ozokerites and synthetic waxes.

Pigments may be included and may be white or coloured, inorganic or organic and/or paerlescent. These pigments comprise titanium dioxide, zinc oxide, ziriconium dioxide, black, yellow, red and brown iron oxides, cerium dioxide, chromium oxide, ferric blue, carbon black, barium, strontium, calcium and aluminum lakes and mica coated with titanium oxide or with bismuth oxide.

Active ingredients commonly employed in skin creams, such as vitamins, for example as vitamin A or C and alpha hydroxy acids, such as citric, glycolic, lactic and tartaric, into cosmetic and/or dermatological compositions may be included. For example, US patent 5,602,183 teaches that vitamin C or ascorbic acid promotes growth of connective tissue, particularly in the skin strengthens the skin against external aggressions such as from smoke and UV radiation. Moisturizing agents which may be included in skin creams and cosmetics are for example mineral oil and urea. Antioxidants such as the naturally occurring tocopherols and polyphenols, or butylated hydroxytoluene and hydroxyanisole may also be also added. Sunscreens such as octyl methoxycinnamate (Parsol MCX), 3-benzophenone (Uvinul M40) and butylmethoxydibenzoylmethane (Parsol 1789) may be employed to prepare a sun tanning lotion. Pharmaceutically active ingredients which may be used to formulate cosmetic compositions include for example antibiotics, fungicides and ant-inflammatory agents.

The final cosmetic product may be in the form of a free, poured or compacted powder (foundation, blusher or eyeshadow), a relatively greasy product such as lipstick, mascara, or an oil or lotion for the body or face.

The washed oil body preparation may also be used to serve as an orally acceptable carrier in toothpaste which may further comprise silicas, surfactants, chelating agents, a fluoride, thickeners, sweeteners, flavorants, for example as oil of peppermint, enzymes and biocides.

An example of an industrial product which may be formulated is paint wherein the main resin, such as those based on silicone type compounds, acrylic compounds, polyester, akyd, fluorine, epoxy, polyurethane may be partly or entirely replaced by the washed oil body preparation of the present invention. Further additives such as pigments, dyes, glass flakes, and aluminum flakes, pigment dispersants, thickeners, levelling agents, hardening catalysts, hardening agents such as dioisocyanates, hardening catalysts, gelling

inhibitors, ultraviolet absorbing agents, free radical quenching agents etc. may be formulated in paint compositions as required.

The washed oil body preparation may also be to formulate lubricants. For example, the washed oil body preparation may be used to partially or entirely replace the lubricating oils such as animal oils, vegetable oils, petroleum lubricating oils, synthetic lubricating oils, or the lubricating grease such as lithium grease, urea grease and calcium grease. Other compositions employed in a lubricant formulation comprise antioxidants, detergent dispersants, oilness agents, friction modifiers, viscosity index improvers, pour point depressants, solid lubricant material, rust inhibitors and antifoamers.

Waxes may also be prepared using the washed oil body preparation of the present invention. These comprise rinse-wax types, such as those providing a stable hydrophobic film-finish onto automobiles and other protective coatings. Other compositions used in the preparation of a wax comprise surfactants, mineral oils, such as mixed paraffinic and aromatic/naphtenic oils, perfumes, biocides, colouring agents which may be added in compatible amounts as desired.

Where industrial products, such as paints or lubricants are formulated, purity of the oil body phase may be less critical and it may not be necessary to subject the oil bodies to washing. An industrial emulsion may be prepared by (i) obtaining oil bodies from a cell and (ii) formulating the oil bodies into an industrial emulsion. The oil bodies may be obtained by (a) grinding plant seeds; (b) removing solids from the ground seeds; and (c) separating the oil body phase from the aqueous phase. The invention also includes an industrial emulsion comprising oil bodies prepared according to the present invention.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

Example 1

Obtaining a washed oil body preparation from oilseed rape, soybean, sunflower, white mustard, peanut, squash, flax, safflower and maize - laboratory scale. Dry mature seeds obtained from *Brassica napus* cv Westar, soybean, sunflower, white mustard, peanut, squash, flax, safflower and maize were homogenized in five volumes of cold grinding buffer (50 mM Tris-HCl, pH 7.5, 0.4 M sucrose and 0.5 M NaCl) using a polytron operating at high speed. The homogenate was centrifuged at 10 x g for 30 minutes in order to remove particulate matter and to separate oil bodies from the aqueous phase containing the bulk of the soluble seed protein. The oil body fraction was skimmed from the surface of the supernatant with a metal spatula and added to one volume of grinding buffer. In order to achieve efficient washing in subsequent steps it was found to be necessary to thoroughly redisperse the oil bodies in the grinding buffer. This was accomplished by gently homogenizing the oil bodies in grinding buffer using a polytron at low speed. Using a syringe, the redispersed oil bodies were carefully layered underneath five volumes of cold

50 mM Tris-HCl pH 7.5 and centrifuged as above. Following centrifugation, the oil bodies were removed and the washing procedure was repeated two times. The final washed oil body preparation was resuspended in one volume of cold Tris-HCl pH 7.5, redispersed with the polytron.

The oil body samples were dissolved in SDS sample buffer and then analyzed by SDS gel electrophoresis. The results are shown in Figure 1.

The material thus obtained material was then ready to be employed in various formulations.

Example 2

Obtaining a washed oil body preparation from oilseed rape, sunflower and maize on a large scale. This example describes the recovery of the oil body fraction from canola, sunflower and maize seed on a large scale. The resulting preparation contains intact oil bodies and is comparable in purity with a preparation obtained using laboratory scale procedures.

Grinding of seeds. A total of 10 - 15 kgs of dry canola seed (*Brassica napus* cv Westar), sunflower (*Helianthus annuus*) or maize (*Zea mays*) was poured through the hopper of a colloid mill (Colloid Mill, MZ-130 (Fryma); capacity: 500 kg/hr), which was equipped with a MZ-120 crosswise toothed rotor/stator grinding set and top loading hopper. Approximately 50 - 75 l water was supplied through an externally connected hose prior to milling. Operation of the mill was at a gap setting of 1R, chosen to achieve a particle size less than 100 micron at 18°C and 30°C. Following grinding of the seeds tap water was added to the seed slurry to a final volume of 90 litres.

Removal of solids. The resulting slurry, was pumped into a decantation centrifuge (Hasco 200 2-phase decantation centrifuge maximum operating speed 6,000 rpm) after bringing the centrifuge up to an operating speed of 3,500 rpm. Transfer from the mill to the decantation centrifuge at a flow rate of 360 L/hr was achieved using a 1 inch M2 Wilden air operated double diaphragm pump. In 15-20 minutes approximately 15 kg of seed was decanted.

Oil body separation. Separation of the oil body fraction was achieved using a Sharples Tubular Bowl Centrifuge model AS-16 (Alpha Laval) equipped with a three phase separating bowl and removable ring dam series; capacity:150 L/hr; ringdam: 30 mm. Operating speed was at 15,000 rpm (13,200 x g). A Watson-Marlow (Model 704) peristaltic pump was used to pump the decanted liquid phase (DL) into the tubular bowl centrifuge after bringing the centrifuge up to operating speed. This results in separation of the decanted liquid phase into a heavy phase (HP) comprising water and soluble seed proteins and a light phase (LP) comprising oil bodies. The oil body fraction which was obtained after one pass through the centrifuge is referred to as an unwashed oil body preparation. The oil body fraction was then passed through the centrifuge three more times. Between

each pass through the centrifuge, concentrated oil bodies were mixed with approximately five volumes of fresh water. The entire procedure was carried out at room temperature. The preparations obtained following the second separation are all referred to as the washed oil body preparation. Following three washes much of the contaminating soluble protein was removed and the oil body protein profiles obtained upon SDS gel electrophoresis were similar in appearance to those obtained using laboratory scale procedures.

Example 3

Removal of seed proteins by washing the oil body phase. This example describes the recovery of a washed oil body fraction from canola, maize and sunflower seed. Using different washing conditions, it is shown that the washes result in the removal of significant amounts of seed proteins from the oil body preparation. These proteins include proteins which might be allergenic.

A total of 10 - 15 kgs of dry canola seed (*Brassica napus* cv Westar), maize (*Zea mays*) or sunflower (*Helianthus annuus*) was poured through the hopper of a colloid mill (Colloid Mill, MZ-130 (Fryma)), which was equipped with a MZ-120 crosswise toothed rotor/stator grinding set and top loading hopper. Approximately 50 - 75 l water was supplied through an externally connected hose prior to milling. Operation of the mill was at a gap setting of 1R, chosen to achieve a particle size less than 100 micron at 18°C and 30°C. Following grinding of the seeds, tap water was added to the seed slurry to a final volume of 60 - 90 litres and a sample of the seed slurry was obtained for SDS gel electrophoresis. The slurry was then pumped into a decantation centrifuge (Hasco 200 2-phase decantation centrifuge maximum operating speed 6,000 rpm) after bringing the centrifuge up to an operating speed of 3,500 rpm. Transfer from the mill to the decantation centrifuge was achieved using a 1 inch M2 Wilden air operated double diaphragm pump. In 15-20 minutes approximately 15 kg of seed was decanted. A sample from the decanted liquid phase was obtained for SDS gel electrophoresis. Separation of the oil body fraction was achieved using a Sharples Tubular Bowl Centrifuge model AS-16 (Alpha Laval) equipped with a three phase separating bowl and removable ring dam series; capacity: 150 L/hr; ringdam: 29 mm. Operating speed was at 15, 000 rpm (13,200 x g). A Watson-Marlowe (Model 704) peristaltic pump was used to pump the decanted liquid phase into the tubular bowl centrifuge after bringing the centrifuge up to operating speed. The unwashed oil body phase was obtained and mixed with approximately 5 volumes of water. This procedure was repeated a total of three more times. The oil body phase which was obtained following the first spin, is referred to as an unwashed oil body preparation. All other preparations are washed oil body preparations. Samples for analysis by SDS gel electrophoresis were obtained following the first and fourth separations.

Upon completion of the fourth wash a 0.9 ml sample of the oil body preparation was homogenized in 0.1 ml 1 M Na_2CO_3 and left at room temperature for 30'

with agitation. The washed oil body fraction was then recovered following centrifugation, washed once with water and prepared for SDS gel electrophoresis.

All of the samples were dissolved in SDS sample buffer and the samples were analyzed by SDS gel electrophoresis. The results are shown in Figure 2.

Example 4

The effect of washing the oil body phase on water retention characteristics. A washed oil body preparation and an unwashed oil body phase were prepared from rapeseed as in example 2. To determine the difference in water retention capacity between the unwashed oil body phase and the washed oil body preparation, 30 mls of oil body preparations were thoroughly mixed using a vortex. The preparations were then incubated for 2 hours in a water bath at 40, 60 or 80°C and the samples were centrifuged at 1,500 x g for 20 minutes (undiluted samples). Another set of samples was prepared by mixing 15 g of washed or unwashed oil body preparation with 15 ml of water. The samples were mixed on a vortex and then incubated at 40, 60 or 80° C for 2 hours and the amount of water present in the samples was determined following centrifugation at 1,500 x g for 20 minutes (diluted samples). Loss of mass attributable to evaporation was measured at 80 °C and 60 °C.

At 80°C, the undiluted preparations comprising oil bodies lost significant amounts of water through evaporation. The preparation of unwashed oil bodies lost 26% of their mass, while the washed preparation lost 16%. Upon centrifugation the unwashed preparation released approximately 2.5 ml of aqueous phase, while the washed oil bodies remained in the same phase. Both diluted preparations absorbed water. The volume of oil bodies increased in both cases to 18.5±1 ml.

At 60°C, the undiluted preparations lost approximately 10% of water through evaporation. Following centrifugation, the washed preparation released about 0.5 ml of aqueous phase, while the washed oil body preparation stayed in the same phase. Both diluted preparations absorbed water. At 60°C, the volume of oil bodies increased in both cases to 18± 1ml.

At 40°C, the undiluted samples both released approximately 2 ml of aqueous phase. When the diluted samples were compared, the unwashed preparation absorbed about 3 ml of water, as was the case at 60 or 80° C. However the washed preparation absorbed 8 ml of water at 40°C.

These experiments demonstrate that in a washed oil body preparation heated to 60°C or 80°C, water remains more tightly associated with the oil body preparation than in an unwashed preparation. When cooled down the washed oil body preparation appeared to be more stable than the unwashed emulsion. When heated to 40°C, the washed oil body preparation was able to absorb a larger volume of exogenously added water without resulting in phase separation.

Example 5

The effect of washing oil bodies on oil absorption characteristics. A washed oil body preparation and an unwashed oil body phase were prepared from rapeseed as in example 2. To determine the difference in oil absorption capacity between the unwashed oil body phase and the washed oil body preparation, 2 gr of the oil body preparations was dispersed into 12 ml of refined, bleached, deodorized canola oil in a 50 ml tube. The contents were stirred for 30 seconds every 5 minutes for 30 min. The tubes were then centrifuged at 4,400 rpm for 25 min. The free oil was decanted and the percentage of absorbed oil was determined by weight difference. Three preparations of washed oil bodies were tested and three preparations of unwashed oil bodies were tested.

The oil absorption capacity of unwashed oil bodies was found to vary significantly between the three batches and varied from 18.7% to 28%. Washed oil bodies had reproducible oil absorption of 32±1%. Thus the washed oil body preparation was found to be superior since (1) a larger amount of oil was found to be absorbed providing and (2) the absorption occurred in a more reproducible manner.

Example 6

Preparation of a mayonnaise-like emulsion comprising a washed oil body preparation. A washed oil body preparation was prepared from rapeseed as in example 2 and a mayonnaise-like emulsion was produced by mixing the following components using a domestic electric blender.

Sunflower oil	78 gr
Egg yolk	8 gr
Vinegar	9 gr
Salt	0.5 gr
Washed oil bodies	5 gr

A product with a mayonnaise-like texture was obtained. The mayonnaise-like product was stable for at least 1 day at 4°C.

Example 7

Preparation of a cholesterol-free mayonnaise-like emulsion. A washed oil body preparation was prepared from rapeseed as in Example 2 and a mayonnaise-like emulsion was produced by mixing the following ingredients:

Sunflower oil	200 gr
Washed oil bodies	100 gr
Vinegar	30 ml

A product with a mayonnaise-like texture was obtained. Since the mayonnaise is prepared without egg yolk, an ingredient commonly employed in commercially obtainable mayonnaises, the product prepared using washed oil bodies is free of cholesterol. The mayonnaise was found to be as stable as a commercial mayonnaise when stability was assessed using centrifugation.

Example 8

Preparation of a vinaigrette-like emulsion comprising a washed oil body preparation. A washed oil body preparation was prepared from rapeseed as in example 2 and a vinaigrette-like emulsion was produced by manual mixing of the following components.

Sunflower oil	17.5 gr
Mustard	0.4 gr
Vinegar	0.5 gr
Washed oil bodies	7.7 gr

A product with a vinaigrette-like texture was obtained. The vinaigrette-like product was stable for at least several days at 4° C.

Example 9

Preparation of a spreadable mustard-like product. A washed oil body preparation was obtained from rapeseed as outlined in example 2. The following ingredients were mixed to obtain a mustard-like product.

Mustard	70 gr
Washed oil bodies	30 gr

The resulting emulsion formulation is a mustard-like product which may easily be spread and has creamier, less gritty taste characteristics than mustard.

Example 10

Preparation of a bechamel-like sauce. A washed oil body preparation was obtained from rapeseed as outlined in example 2. The washed oil body preparation was heated at moderate heat and an equal part of flour was added and mixed with the heated washed oil body preparation. While stirring manually, milk was gradually added to this mixture.

Flour	50 gr
Washed oil bodies	50 gr
Milk	100 ml - 1 l.

A bechamel-like sauce was obtained. The consistency of the sauce may be as desired depending on the amount of milk which is added. Additional flavorants also may be added as required. The absence of hydrogenated fatty acids in this product gives it an advantage over a sauce prepared from common domestic margarine.

Example 11

Preparation of a pharmaceutical emulsion for coating onto fish food. A washed oil body preparation from a transgenic *B. napus* plant which expresses carp growth hormone (cGH) fused to oleosin, wherein the fusion protein was targeted to the oil bodies, was obtained as follows. A DNA fragment containing the cGH coding region lacking its 22 amino acid signal sequence was amplified from a plasmid containing on an insert a common carp (*Cyprinus carpio*) growth hormone cDNA (Koren et al., 1989, Gene 67: 309-315) using the polymerase chain reaction in combination with two cGH specific primers. The amplified cGH fragment was fused in the correct reading frame and 3′ to the *Arabidopsis thaliana* oleosin using pOThromb (van Rooijen, 1993, PhD Thesis, University of Calgary) as a parent plasmid and employing cloning strategies known to a person skilled in the art. In pOThromb a thrombin cleavage site was engineered 3′ to the oleosin coding sequence. The oleosin-CGH fusion gene was introduced into the binary vector pCGN1559 (McBride and Summerfelt, 1990, Plant Mol. Biol. 14: 269-276) and the resulting construct was used to transform *A. tumefaciens*. The agrobacterium strain was employed to transform *B. napus* cv Westar seedlings. Seeds from transgenic plants were obtained and oil bodies were isolated from transgenic seed as outlined in example 1.

The oil bodies were subsequently taken up in a syringe and sprayed onto fish food using approximately 2.5 µg of oil body protein per 1 mg of fish food. The fish food coated with oil bodies was then left overnight to dry. A total of 50 mg of fish food was then mixed with 10 ml of water and was incubated for 0, 30, 45 or 60 minutes. The food was then collected and resuspended in 0.2 ml of 50 mM Tris-Cl (pH 7.5) and prepared for analysis by SDS gel electrophoresis upon boiling in 2.5% SDS. The presence of the oil bodies on the fish food was assessed using Western blotting and monoclonal antibodies against cGH.

Judging by the intensity of the signal of the single band observed in each lane on the Western blot, the oil bodies comprising cGH remained tightly associated with the fish food upon incubation of the oil bodies in water. Fish food which was incubated for 30, 45 or 60 minutes in water was shown to contain approximately the same amounts of cGH as the control fish food which was not incubated in water.

This example demonstrates that a transgenic plant variety can be prepared which imparts specific desirable properties to an emulsion. The example further demonstrates that an emulsion can be prepared from a washed oil body preparation which can be used as a coating or film. Finally, this example demonstrates that the washed oil body preparation may be employed to formulate a pharmaceutical composition.

Although certain preferred embodiments have been described to illustrate the present invention, other applications which also fall within the scope of the invention will be readily apparent to those of ordinary skill in the art.

We Claim:

1. A method for preparing an emulsion formulation comprising:
(1) obtaining oil bodies from a cell;
(2) washing the oil bodies to obtain a washed oil body preparation; and
(3) formulating the washed oil body preparation into an emulsion.

2. A method according to claim 1 wherein said cell is a plant cell.

3. A method according to claim 2 wherein said plant cell is obtained from spores, pollen, seed or a vegetative plant organ.

4. A method according to claim 2 wherein the oil bodies are obtained from plant seeds.

5. A method for preparing an emulsion formulation comprising:
(1) obtaining oil bodies from plant seeds by a method that comprises:
(a) grinding plant seeds;
(b) removing solids from the ground seeds; and
(c) separating the oil body phase from the aqueous phase;
(2) washing the oil body phase to yield a washed oil body preparation; and
(3) formulating the washed oil body preparation into an emulsion.

6. A method according to claim 5 wherein a liquid phase is added prior to or following the grinding step.

7. A method according to any one of claims 1 to 6 wherein said formulating comprises mixing the washed oil bodies until an emulsion is formed.

8. A method according to claim 7 wherein a liquid phase is added to the washed oil bodies and the liquid phase and washed oil bodies are mixed until an emulsion is formed.

9. A method according to claim 6 or 8 wherein said liquid phase is water.

10. A method according to claim 9 wherein said liquid phase is present in the emulsion in an amount varying from about 1% to about 99% v/v.

11. A method according to claim 4 or 5 wherein said plant is an oil seed plant.

12. A method according to claim 4 or 5 wherein said plant is selected from the group of plants consisting of rapeseed (*Brassica* spp.), soybean (*Glycine max*), sunflower (*Helianthus annuus*), oil palm (*Elaeis guineeis*), cottonseed (*Gossypium* spp.), groundnut (*Arachis hypogaea*), coconut (*Cocus nucifera*), castor (*Ricinus communis*), safflower (*Carthamus tinctorius*), mustard (*Brassica* spp. and *Sinapis alba*), coriander, (*Coriandrum sativum*), squash (*Cucurbita maxima*), linseed/flax (*Linum usitatissimum*), Brazil nut (*Bertholletia excelsa*) jojoba (*Simmondsia chinensis*) and maize (*Zea mays*).

13. A method according to claim 5 wherein said solids are removed by centrifugation or filtration of said ground seeds.

14. A method according to claim 5 wherein said oil body phase is separated from said aqueous phase by a gravity based method or a size exclusion based method.

15. A method according to claim 5 wherein the washed oil body preparation is essentially free of non-oil body seed proteins, antinutritional compounds, starch, glucosinilates or breakdown products thereof, and fibres.

16. A method according to claim 15 in which said seed proteins are glycosylated.

17. A method according to any one of claims 1 to 16 in which said formulating comprises the addition of a preservative agent.

18. A method according to any one of claims 1 to 16 in which said emulsion is a food or feed product, a personal care product, a pharmaceutical product or an industrial product.

19. A method according to claim 5 in which the seed is immersed in a liquid phase prior to grinding for from approximately 15 minutes to approximately 2 days.

20. An emulsion comprising a washed oil body preparation.

21. An emulsion according to claim 20 further comprising a liquid phase.

22. An emulsion according to claim 21 wherein said liquid phase is water.

23. An emulsion according to claim 20 further comprising a preservative.

24. An emulsion according to claim 20 wherein the oil bodies are obtained from a plant.

25. An emulsion according to claim 24 wherein the oil bodies are obtained from a plant selected from the group consisting of rapeseed (*Brassica* spp.), soybean (*Glycine max*), sunflower (*Helianthus annuus*), oil palm (*Elaeis guineeis*), cottonseed (*Gossypium* spp.), groundnut (*Arachis hypogaea*), coconut (*Cocus nucifera*), castor (*Ricinus communis*), safflower (*Carthamus tinctorius*), mustard (*Brassica* spp. and *Sinapis alba*), coriander, (*Coriandrum sativum*), squash (*Cucurbita maxima*), linseed/flax (*Linum usitatissimum*), Brazil nut (*Bertholletia excelsa*) jojoba (*Simmondsia chinensis*) and maize (*Zea mays*).

26. An emulsion according to any one of claims 20 to 25 wherein said emulsion is a food product, a personal care product, a pharmaceutical product or an industrial product.

27. An emulsion according to claim 26 wherein said food or feed product is selected from the group consisting of a non-dairy substitutes, a non-dairy cheese, a non-dairy yoghurt, a margarine, a mayonnaise, a vinaigrette, an icing, ice creams, a salad dressing, a synthetic mustard, a candy, chewing gum, a pudding, a baking product, a condiment, a juice, baby formula, a flavour carrier, a texturing agent, petfood and livestock feed.

28. An emulsion according to claim 26 wherein said food or feed product is a mayonnaise, a cholesterol free mayonnaise, a bechamel sauce, a vinaigrette, a mustard or a fish food.

29. An emulsion according to claim 26 wherein said personal care product is selected from the group consisting of a soap, a cosmetic product, a skin cream, a facial cream, a tooth paste, a lipstick, a perfume, make-up, a foundation, a blusher, a mascara, an eyeshadow, a sunscreen lotion and a hair care product.

30. An emulsion according to claim 26 wherein said pharmaceutical product is selected from the group consisting of a therapeutic agent, a diagnostic agent and a delivery agent.

31. An emulsion according to claim 30 wherein said therapeutic agent includes a growth hormone.

32. An emulsion according to claim 26 wherein said industrial product is selected from the group consisting of a paint, a coating, a lubricant, a film, a gel, a drilling fluid, paper sizing, a latex, a building or road construction material, an ink, a dye, a wax, a polish and an agrochemical formulation.

33. A use of an emulsion prepared according to any one of claims 1 to 19 to prepare a personal care product, a food or feed product, an industrial product or a pharmaceutical product.

34. A use of an emulsion according to claim 33 wherein said food or feed product comprises a non-dairy substitutes, a non-dairy cheese, a non-dairy yoghurt, a margarine, a mayonnaise, a vinaigrette, an icing, ice creams, a salad dressing, a synthetic mustard, a candy, chewing gum, a pudding, a baking product, a condiment, a juice, baby formula, a flavour carrier, a texturing agent, petfood and livestock feed.

35. A use of an emulsion according to claim 33 wherein said food or feed product is a mayonnaise, a cholesterol free mayonnaise, a bechamel sauce, a vinaigrette, a mustard or a fish food.

36. A use of an emulsion according to claim 33 wherein said personal care product comprises a soap, a cosmetic product, a skin cream, a facial cream, a tooth paste, a lipstick, a perfume, make-up, a foundation, a blusher, a mascara, an eyeshadow, a sunscreen lotion, a hair care product.

37. A use of an emulsion according to claim 33 wherein said industrial product comprises a paint, a coating, a lubricant, a film, a gel, a drilling fluid, paper sizing, a latex, a building or road construction material, an ink, a dye, a wax, a polish and an agrochemical formulation.

38. A use of an emulsion according to claim 33 wherein said pharmaceutical product is a therapeutic agent, a diagnostic agent or a delivery agent.

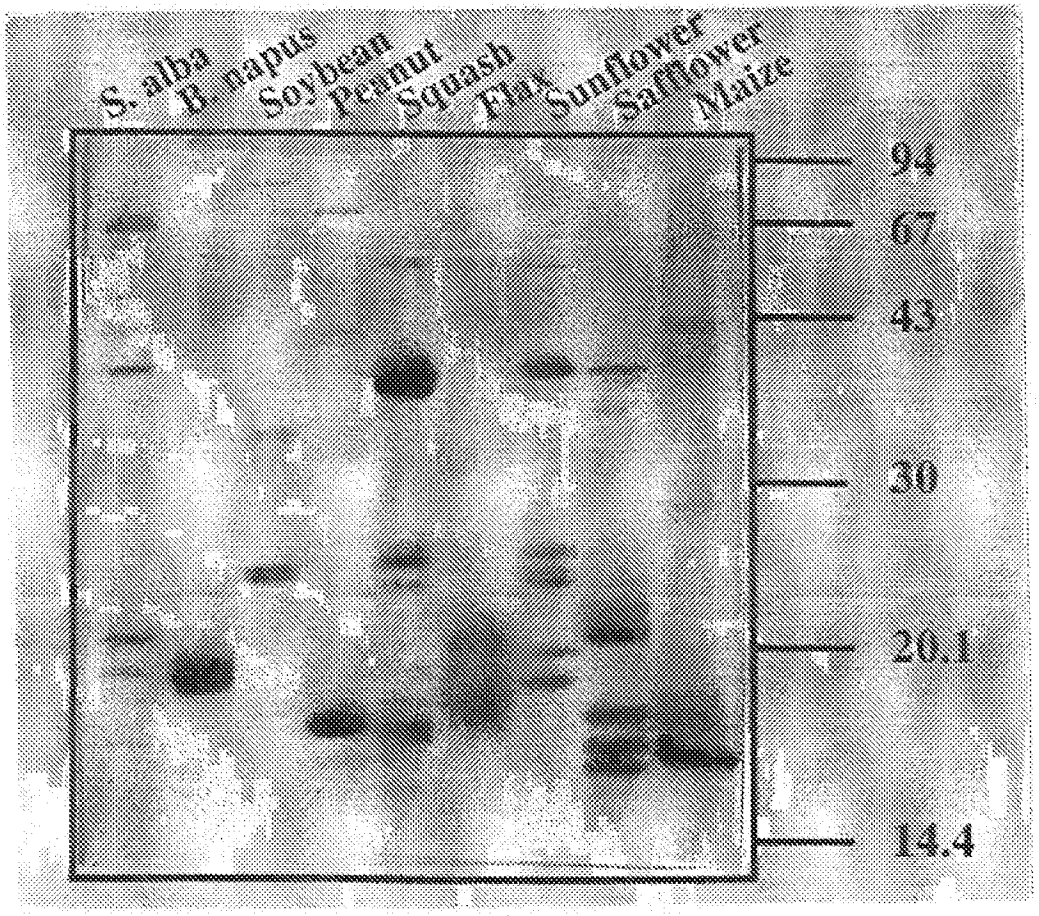
39. A use of an emulsion prepared according to any one of claims 1 to 19 as a coating, a film or an aerosol.

40. A fish food preparation coated with an emulsion according to any one of claims 20 to 25.

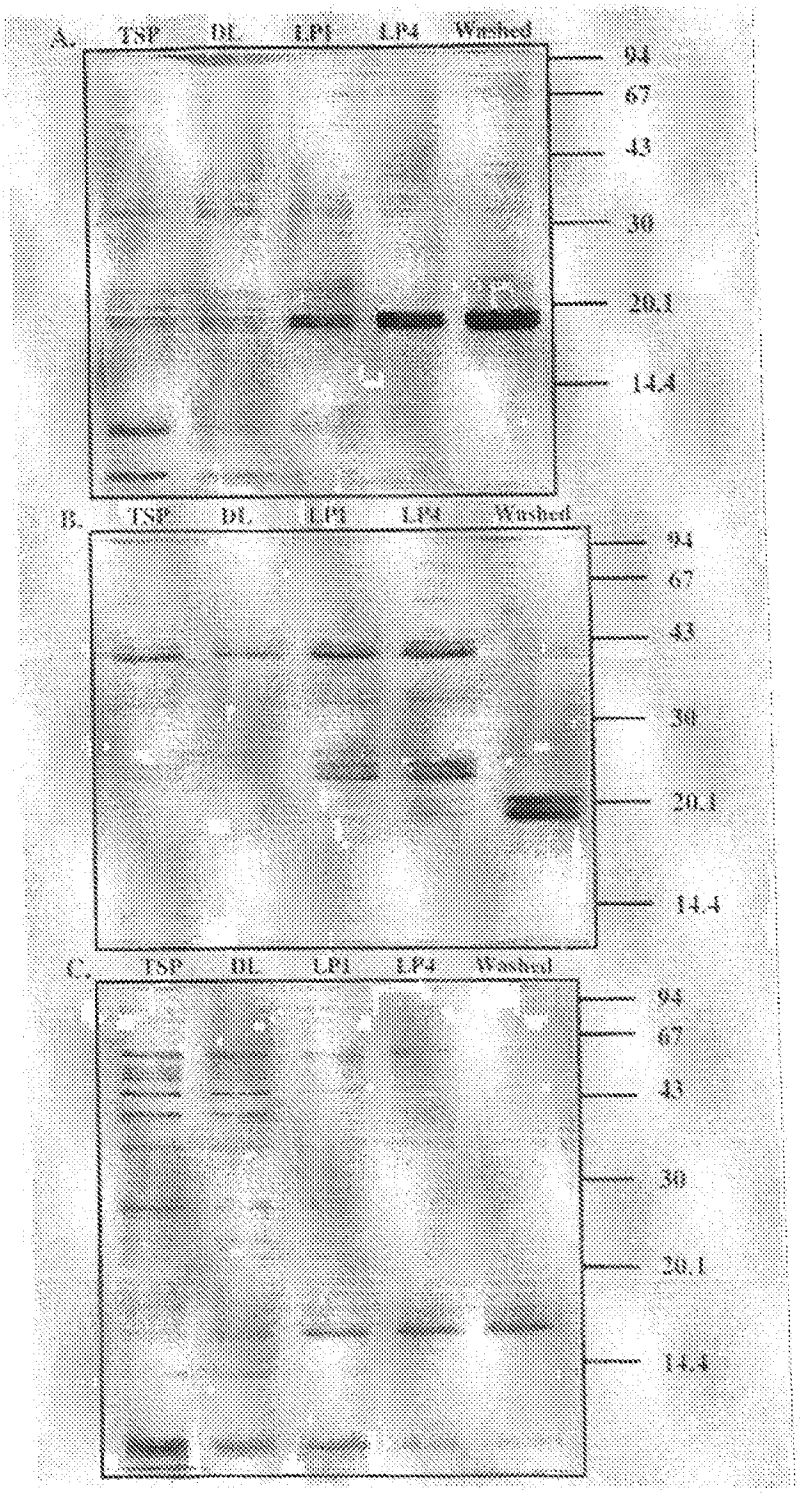
41. A fish food preparation according to claim 40 wherein said emulsion additionally contains a therapeutic peptide.

42. A fish food preparation according to claim 41 wherein said therapeutic peptide is a hormone.

1/2
FIGURE 1


S. alba
B. napus
Soybean
Peanut
Squash
Flax
Sunflower
Safflower
Maize
94
67
43
30
20.1
14.4

2/2
FIGURE 2


A.
TSP
DL
LP1
LP4
Washed
94
67
43
30
20.1
14.4
B.
TSP
DL
LP1
LP4
Washed
94
67
43
30
20.1
14.4
C.
TSP
DL
LP1
LP4
Washed
94
67
43
30
20.1
14.4

INTERNATIONAL SEARCH REPORT

In tional Application No
PCT/CA 98/00523

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A23D7/00 C11B1/10 B01F17/00 A23L1/035 A23K1/16
A23L1/24 A23L1/39 - A61K7/00 A61K9/107

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C11B A23D A23L A23K A61K B01F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 680 751 A (ROCHER YVES BIOLOG VEGETALE) 8 November 1995 see page 2, column 1, line 33 - page 5, column 7, line 36 see claims 1,3,4,7-10	1-14,17, 18, 20-27, 29,30, 33,34, 36,38
Y	--- -/--	15,16, 28,31, 32,35,37

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

° Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
23 October 1998	05/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Dekeirel, M

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 98/00523

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No
Y	M. MILLICHIP ET AL.: "Purification and characterization of oil-bodies (oleosomes) and oil-body boundary proteins (oleosins) from the developing cotyledons of sunflower (Helianthus annuus L.)" BIOCHEMICAL JOURNAL, vol. 314, no. 1, 1996, pages 333-337, XP002081853 LONDON, GB see page 333, Abstract see page 334, column 1, paragraph 2 see page 334, column 2, paragraph 6 - page 335, column 1, paragraph 1 see page 336, column 2, paragraph 2 - page 337, column 2, paragraph 1 ---	15,16
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(54) Title: ENHANCEMENT OF CELL GROWTH BY EXPRESSION OF CLONED OXYGEN-BINDING PROTEINS

(57) Abstract

Methods for the use of DNA encoding oxygen-binding proteins and related plasmids containing same are disclosed for a range of applications including oxygen supply to cells, growth enhancement, expression of various gene products, enhancement of oxygen-requiring processes, binding and separation of oxygen from liquids and gases, and a range of oxidative reactions.

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ENHANCEMENT OF CELL GROWTH BY EXPRESSION OF CLONED OXYGEN-BINDING PROTEINS

The United States Government has rights in this invention pursuant to Grant No. BCS-8805636 from the National Science Foundation.

This is a continuation-in-part of Ser. No. 07/887169, filed May 20, 1992, which is a continuation-in-part of Serial No. 07/741,789, filed August 6, 1991, which is a continuation of Serial No. 342,451, filed October 21, 1988, now U.S. 5,049,493, which is a continuation-in-part of Serial No. 151,256, filed February 28, 1988, abandoned, and of Serial No. 113,014, filed October 23, 1987, abandoned, all of which are incorporated by reference herein.

TECHNICAL FIELD

This invention relates to the production of oxygen-binding proteins, particularly members of the globin family, and to enhancement of the growth and product synthesis characteristics of aerobic organisms in environments with sufficient as well as reduced or low levels of oxygen.

This invention relates generally to the use of recombinant DNA technology to direct or otherwise control gene expression in cultured cells, and more particularly, to methods and materials useful in subjecting the transcription and translation of DNA

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sequences to selective regulation by external control.

BACKGROUND ART

The present invention is directed toward the expression and use of oxygen-binding proteins, including, but not limited to, the evolutionary-related globin superfamily: hemoglobins (found in many eukaryotic organisms); leghemoglobins (found in many plants); myoglobins (found mainly in animal muscle tissue); and microbial globins such as Vitreoscilla hemoglobin; E. coli hmp (a flavohemoglobin), and yeast flavohemoglobin. Other oxygen-binding hemoproteins include cytochrome C reductase, cytochrome c oxidase, and kinase in the oxygen sensor of Rhizobium meliloti. Additional oxygen-binding proteins which function in the transport of oxygen in invertebrates include hemocyanins and hemerythrins. The existence of Vitreoscilla hemoglobin, E. coli hmp (Mol. Gen. Genet. 226: 49-58, 1991) and yeast flaxohemoglobin (F.A.S.E.B. J. 6: A318, 1992), incorporated herein by reference, suggests that globin-like oxygen-binding proteins may also be distributed in microrganisms. Such endogenous, homologous globins may also be utilized in accordance with the present invention, providing the added advantage of expressing a globin from a gene homologous to the desired host organism. By reversibly binding to oxygen in the presence of high oxygen concentrations and releasing it in regions or at times of low concentrations, these oxygen-binding proteins can considerably enhance the oxygen uptake rate of multicellular organism over that allowed by mere passive diffusion. In unicellular organisms it is generally believed that the oxygen uptake rate is principally limited by

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the rate of transfer of dissolved oxygen in the environment or growth medium to the exterior cell surface. However, closer examination of cell structure reveals several potential diffusional barriers between environmental oxygen and the cytochromes where the oxygen finally undergoes reaction. For example, in gram negative bacteria, where the cytochromes are attached to the inside of the plasma membrane, the diffusing oxygen needs to cross transport barriers such as the cell wall, the outer membrane, the periplasmic space and the inner membrane before accepting electrons from metabolic reactions. In unicellular eucaryotes, where oxidative phosphorylation takes place in the mitochondria, there are further diffusional resistances. Small neutral molecules like oxygen are assumed to passively diffuse across these barriers; however, these barriers make a non-trivial contribution to the overall resistance to mass transfer to the actual reaction site and thus could be of significance under conditions of low oxygen or oxygen limitation.

Physiological effects on growth due to depletion in dissolved oxygen levels has been demonstrated in the case of several organisms, including Escherichia coli, Saccharomyces cerevisiae, Pseudomonas strains, and Alcaligenes eutrophus. In E. coli for example, which has a very high affinity cytochrome, changes in dissolved oxygen tension leads to differential regulation of terminal oxidases, resulting in a decrease in the number of protons expelled per NADH molecule oxidized during aerobic respiration and, consequently, a possible adverse change in the stoichiometry of ATP biosynthesis. (Kranz et al., Journal of Bacteriology 158:1191-1194, 1984; Ingraham et al., Growth of the bacterial cell, Sinauer

Associates, Inc. 1983, p. 147, both specifically incorporated herein.)

In addition to the respiratory oxygen requirement of aerobic organisms, oxygen-binding proteins have other potential applications as well, including, for example, the enhancement of particular oxidative transformations such as steroid conversions, vinegar production, biological waste treatment or enzymatic degradations, and in some steps in brewing or making distilled and fermented foods and beverages.

The filamentous bacterium, Vitreoscilla, a member of the Beggiatoa family, is a strict aerobe that is found in oxygen-poor environments such as stagnant ponds and decaying vegetable matter. Growth of the bacterium under hypoxic conditions results in a several-fold induction of synthesis of a homodimeric soluble heme protein (subunit MW 15,775) (Boerman et al., Control of heme content in Vitreoscilla by oxygen, Journal of General Applied Microbiology 28:35-42, 1982) which has a remarkable spectral (Webster, et al., Reduced nicotinamide adenine dinucleotide cytochrome o reductase associated with cytochrome o purified from Vitreoscilla, Journal of Biological Chemistry 249:4257-4260, 1974), structural (Wakabayashi, et at., Primary sequences of a dimeric bacterial hemoglobin from Vitreoscilla, Nature 322:481-483, 1986), and kinetic (Orii, et al., Photodissociation of oxygenated cytochrome o(s) (Vitreoscilla) and kinetic studies of reassociation, Journal of Biological Chemistry 261:2978-2986, 1986) homology with eucaryotic hemoglobins, and which is probably a true bacterial hemoglobin.

This protein was previously thought to be a cytochrome o, and it has been suggested to function

in oxygen storage. However, biochemical discrepancies (Webster, et al., Oxygenated cytochrome o, Journal of Biological Chemistry 252:1834-1836, 1977) as well as the subsequent discovery of the true membrane-bound cytochromes o and d (DeMaio, et al., Spectral evidence for the existence of a second cytochrome o in whole cells of Vitreoscilla, Journal of Biological Chemistry 258:13768-13771, 1983; Webster et al., Federation Proceeding 44:678, 1985) led to further investigations of its spectral properties (Choc et al., Oxygenated intermediate and carbonyl species of cytochrome o (Vitreoscilla), Journal of Biological Chemistry 257: 865-869, 1982; Orii et al., supra.) and the eventual determination of its probable amino acid sequences and partial homology with known hemoglobin sequences (23).

Although these articles disclose the conservation of most features characteristic of eukaryotic hemoglobins, and discuss, to some extent, the role or potential role it probably plays in oxygen utilization, it is not believed to have been suggested by others that there is any benefit from the introduction of a bacterial hemoglobin or other oxygen-binding proteins in heterologous organisms. Moreover, it is believed that there has been no suggestion that such oxygen-binding proteins would have a far-reaching range of applications.

Surprisingly, the present inventors have discovered that oxygen-binding proteins are useful in enhancing oxygen supply to cells or in other oxygen-utilizing processes, and for binding and separating oxygen from other fluids or gases. Furthermore, the oxygen-binding proteins are capable of increasing production of cells, or of proteins or metabolites normally made by a cell, or of natural or unnatural metabolites and

proteins expressed in a cell via genetic manipulation. These proteins are also useful as selective markers in recombinant-DNA work, and have applications as diverse as enhancing certain oxygen-requiring steps in fermentation, enzymatic degradation, toxic chemical waste treatment, brewing, and particular oxidative reactions and transformations.

A preferred method of expressing these proteins in bacteria is to use the promoter/regulator sequence of V. hemoglobin of control expression of the homologous oxygen-binding protein. The DNA sequences which usually precede a gene in a DNA polymer and which provide a site for initiation of the transcription of that gene into mRNA. These are referred to as "promoter" sequences. Other DNA or RNA sequences, also usually but not necessarily "upstream" of a structural gene, bind proteins that determine the frequency or rate of transcription and/or translation initiation. These other sequences, including attenuators, enhancers, operators and the like, are referred to as "regulator" sequences. Thus, sequences which operate to determine whether the transcription and eventual expression of a gene will take place are collectively referred to as "promoter/regulator" DNA sequences.

The lactose ("lac") promoter/operator systems have also been commonly used, for they are very controllable through the mode of action of the operator. When the operator is repressed, the DNA dependent RNA polymerase is completely prevented from binding and initiating transcription, thus effectively blocking promoter operability. This system can be derepressed by induction following the addition of a known inducer, such as isopropyl-beta-

D-thiogalactoside (IPTG). The inducer causes the repressor protein to fall away so the RNA polymerase can function.

Cells transformed with plasmids carrying the lac promoter/operator system can be permitted to grow up to maximal density while in the repressed state through the omission of an inducer, such as IPTG, from the media. When a high level of cell density is achieved, the system can be derepressed by addition of inducer. The promoter is then free to initiate transcription and thus obtain expression of the gene products at yields commensurate with the promoter strength. However, certain of these inducible promoter systems are relatively weak and commercial or research productions using such systems do not urge the cell to generate maximum output.

In response to the need for microbial expression vehicles capable of producing desired products in higher yield, the tryptophan ("trp") promoter/operator system has become widely used. This system is one of several known systems with at least three times the strength of the lac promoter. However, it has the disadvantage of less promoter control. The trp promoter is not inducible in the way the lac promoter is, namely, the bound repressor is not removed by induction. Instead, the system operates on a sort of feedback loop as described above. A system was devised whereby the attenuator region of the trp promoter/operator system was removed, with the resultant transformed cells being grown in tryptophan-rich media. This provided sufficient tryptophan to essentially completely repress the operator so that cell growth could proceed uninhibited by premature expression of any desired foreign proteins. When the culture reached

appropriate growth levels, no additional tryptophan was supplied, resulting in mild tryptophan limitation, and, accordingly, derepression of the promoter with resultant expression of the desired protein gene insert. In application, this system has several disadvantages. For example; it is necessary to maintain high levels of tryptophan in the growth media to completely repress the promoter, and to permit the medium to become completely exhausted of tryptophan following full growth of the culture.

A hybrid system has been developed from the tryptophan and lactose promoter, wherein both promoters can be repressed by the lac repressor and both can be derepressed with IPTG. See De Boer et al., The tac promoter: A functional hybrid derived from the trp and lac promoters, Proc. Natl. Acad. Sci. USA, 80: 21-25, 1983. This system shares a disadvantage with the two discussed above, namely the required introduction of additional agents to a normal growth medium.

Another regulator/promoter system commonly used for expression of cloned proteins in E. coli is based on the P_L promoter system from phage lambda. See Bernard and Helsinki, Methods in Enzymology, 68:482-492, 1979; Use of Lambda Phage Promoter P_L to Promote Gene Expression In Hybrid Plasmid Cloning Vehicles. Induction of this promoter requires increase of culture temperature from 30°C to 42°C. This system has the disadvantages of suboptimal growth rates at 30°C prior to induction and upsetting of cell metabolism by the temperature shift. Temperature shift effects on metabolism are discussed, for example, by Neidhart, et. al., The Genetics And Regulation Of Heat-Shock Proteins, Annual Reviews of Genetics, 18:295-329, 1984.

The promoter systems described above are thus useful for expression of proteins of the globin family.

DISCLOSURE OF THE INVENTION

The present invention relates to the expression and use of oxygen-binding proteins, particularly hemoglobins, recombinant-DNA methods of producing same, and to portable DNA sequences capable of directing intracellular production of these oxygen-binding proteins.

The present invention provides novel methods and materials for subjecting DNA sequences of living microorganisms to external regulation which is dependent upon availability of oxygen in the environment. Particularly, it relates to promoter/regulators, a recombinant-DNA method of producing same, and to portable DNA sequences capable of directing the translation and transcription initiation and control of the expression of desired gene products.

Thus, another object of the present invention is provide for the control of expression of any selected chromosomal or extrachromosomal gene or DNA sequence through the incorporation of a promoter/regulator DNA sequence which is functionally responsive to environmental variations in the concentration of oxygen. The invention is thus broadly applicable to a variety of aerobic or slightly aerobic procedures for controlling genetic processes, ranging from the alteration of existing regulation of endogenous genes in prokaryotic and eucaryotic cells to securing selective, differential regulation of expression of selected exogenous or foreign genes stably incorporated in host cells.

Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description or may be learned from practice of the invention. The objects and advantages may be realized and attained by means of the instrumentalities and combination particularly pointed out in the appended claims.

To achieve the objects and in accordance with the purposes of the present invention, oxygen-binding proteins are set forth which are capable of stoichiometric reaction with oxygen. To further achieve the objects and in accordance with the purposes of the present invention, as embodied and broadly described herein, portable DNA sequences coding for hemoglobin proteins are provided.

Particularly preferred for expression in bacteria, portable sequences are provided which code for the hemoglobin of the filamentous bacterium Vitreoscilla. These sequences comprise nucleotide sequences capable of directing intracellular production of oxygen-binding proteins. The portable sequences may be either synthetic sequences or restriction fragments ("natural" DNA sequences).

To facilitate identification and isolation of natural DNA sequences for use in the present invention, the inventors have developed a Vitreoscilla genomic library. This library contains the genetic information capable of directing a cell to synthesize the hemoglobin of the present invention. Other natural DNA sequences which may be used in the recombinant DNA methods set forth herein may be isolated from other genomic libraries.

Additionally, portable DNA sequences useful in the processes of the present invention may be synthetically created. These synthetic DNA sequences may be prepared by polynucleotide synthesis and sequencing techniques known to those of ordinary skill in the art.

Additionally, to achieve the objects and in accordance with the purposes of the present invention, a recombinant-DNA method is disclosed which results in manufacture by a host cell or microorganism of the instant oxygen-binding proteins using the portable DNA sequences referred to above. This recombinant-DNA method comprises:

(a) preparing a portable DNA sequence capable of directing a host cell to produce a protein having oxygen-binding activity, including hemoglobin activity;

(b) cloning the portable DNA sequence directly into a host cell, or into a vector capable of being transferred into and replicating in a host cell, such vector containing operational elements for the portable DNA sequence;

(c) transferring the vector, if one is used, containing the portable DNA sequence and operational elements into a host cell capable of expressing the oxygen-binding protein; and

(d) culturing the host cell under conditions appropriate for replication and propagation of the vector and expression of the protein; and

(e) in either order:

(i) harvesting the protein; and

(ii) permitting the protein to assume an active structure whereby it possesses oxygen-binding activity.

Additionally, to achieve the objects and in accordance with the purposes of the present invention, recombinant-DNA methods are disclosed which subject to external control the translation and transcription of gene products by a host cell or microorganism using the portable DNA sequences referred to above.

Processes of the invention include methods for subjecting the expression of a selected DNA sequence in a living cell or virus to regulation by oxygen level through the site-specific insertion of promoter/regulator DNA sequences responsive thereto. Also disclosed are improvements in prior methods for securing expression of a selected "foreign" or exogenous sequence in a host microorganism wherein the DNA sequence is stably incorporated as chromosomal or extrachromosomal constituent of the host. Such improvements comprise fusing to the selected DNA sequence a promoter/regulator DNA sequence capable of selectively promoting or inhibiting expression of the selected DNA in response to variations in environmental concentration of oxygen.

To further accomplish the objects and in further accord with the purposes of the present invention, cloning vectors are provided comprising at least one portable DNA sequence. In particular, plasmid pUC19/pRED2 is disclosed.

It is understood that both the foregoing general description and the following detailed description

are exemplary and explanatory only and are not restrictive of the invention, as claimed.

The accompanying drawing, which is incorporated in and constitutes a part of this specification, illustrates one embodiment of the invention and, together with the description, serves to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a partial restriction map of the plasmid pUC19/pRED2.

Figs. 2a and 2b are partial restriction maps of plasmids pWLD 5 and pWLD 10, respectively.

Fig. 3 is a partial restriction map of plasmid pBHb3.

Figs. 4A, B, and C and 5A, B, and C show the total cell number, total t-PA and t-PA produced/10^6 cells according to Example 18.

Fig. 6 is a partial restriction map of plasmid pMSG.

Fig. 7 is a graph of results described in Example 37.

BEST MODES FOR CARRYING OUT THE INVENTION

Reference will now be made in detail to the presently preferred embodiments of the invention, which, together with the drawings and the following examples, serve to explain the principles of the invention.

It must be understood that the present inventors have prepared oxygen-binding proteins in culture by recombinant DNA methods. While some methods for the production and use of these recombinant products are

described below, the end use of these products alone is within the scope of the present invention.

As noted above, the present invention relates in part to portable DNA sequences capable of directing intracellular production of oxygen-binding proteins in a variety of host cells and host microorganisms. "Portable DNA sequence" in this context is intended to refer either to a synthetically produced nucleotide sequence or to a restriction fragment of a naturally occurring DNA sequence. For purposes of this specification, "oxygen-binding protein" is intended to mean a protein with a primary structure as defined by the codons present in the deoxyribonucleic acid sequence which directs intracellular production of the amino acid sequence, and which may or may not include post-translational modifications. It is contemplated that such post-translational modifications include, for example, association with a heme prosthetic group. It is further intended that the term "oxygen-binding protein" refers to either the form of the protein as would be excreted from a cell or as it may be present in the cell from which it was not excreted.

In a preferred embodiment, the portable DNA sequences are capable of directing intracellular production of hemoglobin. In a particularly preferred embodiment, the portable DNA sequences are capable of directing intracellular production of a hemoglobin biologically equivalent to that previously isolated from the filamentous bacterium, Vitreoscilla. By "biologically equivalent", as used herein, it is meant that a protein, produced using a portable DNA sequence of the present invention, is capable of binding oxygen in the same fashion, but not necessarily to the same degree, as the homodimeric

soluble heme protein (subunit MW 15,775) isolable from Vitreoscilla.

As noted above, the present invention also relates in part to portable DNA sequences which contain promoter/regulators which are capable of directing intracellular expression of endogenous or exogenous gene products, in a variety of host cells and host microorganisms. "Portable DNA sequence" and "promoter/regulator" in this context are intended to refer either to a synthetically produced nucleotide sequence or to a restriction fragment of a naturally occurring DNA sequence.

The portable DNA sequences of the present invention may also include DNA sequences downstream from a promoter/regulator which code for at least one foreign protein. For purposes of this specification, "foreign protein" is intended to mean a protein with a primary structure as defined by the codons present in the deoxyribonucleic acid sequence which directs intracellular production of the corresponding amino acid sequence, and which may or may not include post-translational modifications. It is further intended that the term "foreign protein" refers to either the form of the protein as it would be excreted from a cell or as it may be present in the cell from which it was not excreted. The foreign proteins include oxygen-binding proteins such as hemoglobins, leghemoglobins, myoglobins, hemoproteins, hemocyanias, hemerythrins and the like.

In a particularly preferred embodiment, the promoter/regulator contains transcription and translation initiation and control sequences substantially equivalent to those for directing intracellular production of a hemoglobin protein

biologically equivalent to that previously isolated from the filamentous bacterium, Vitreoscilla. By "substantially equivalent", as used herein, is meant that a promoter/regulator operates to express a downstream gene product upon reduction of the level of oxygen available to the host cell below some critical value.

It is of course intended that the promoter/regulators of the present invention may control and initiate transcription and translation of an unlimited number of endogenous and/or exogenous foreign proteins.

A first preferred portable DNA sequence for the promoter/regulators of the present invention contains at least a portion of SEQUENCE ID No. 1, a nucleotide sequence, which reads 5' to 3' and includes the translation initiation sequence ATG and some of the nucleotide sequence of the Vitreoscilla structural gene

The SEQUENCE ID No. 1 exhibits homology with certain sequences which are highly conserved in a variety of promoter/regulators. Using conventional numbering, with the underlining showing the homology in the SEQUENCE ID No. 1 to the consensus sequence, the -10 consensus sequence or Pribnow box sequence is TATAAT(A/G). The -35 consensus sequence is TTGACA, and the consensus Shine-Dalgarno sequence is AGGAGGTXXX(XX)ATG.

In a preferred embodiment, the SEQUENCE ID No. 1 is operatively fused with at least a portion of a downstream sequence of nucleotides which code for at least a portion of the Vitreoscilla hemoglobin protein which contains at least a portion of the amino acid sequence of SEQUENCE ID No. 2

The SEQUENCE ID No. 2 is disclosed in Wakabayashi et al., supra, Nature 322:483, 1986. It is presently believed that the protein purified and prepared through the practice of this invention will exhibit a homology of over 80% with this sequence. The protein of this invention has been observed to enhance functioning of a cell in low oxygen environments.

It must be borne in mind in the practice of the present invention that the alteration of some amino acids in a protein sequence may not affect the fundamental properties of the protein. Therefore, it is also contemplated that other portable DNA sequences, both those capable of directing intracellular production of identical amino acid sequences and those capable of directing intracellular production of analogous amino acid sequences which also possess oxygen-binding activity, are included within the ambit of the present invention.

It must also be borne in mind in the practice of the present invention that the alteration of some nucleotide bases in a DNA sequence may not affect the fundamental properties of the coding sequence. Therefore, it is also contemplated that other analogous portable DNA promoter/regulator sequences which are operable through changes in oxygen level are included within the ambit of the present invention.

It is contemplated that some of these analogous amino acid sequences will be substantially homologous to native Vitreoscilla hemoglobin while other amino acid sequences, capable of functioning as oxygen-binding proteins, will not exhibit substantial homology to native Vitreoscilla hemoglobin. By "substantial

homology" as used herein, is meant a degree of homology to native Vitreoscilla hemoglobin in excess of 50%, preferably in excess of 80%.

Similarly, it is contemplated that some of these analogous DNA sequences will be substantially homologous to the sequence set forth above, while other DNA sequences, capable of functioning as the promoter/regulator described above, will not exhibit substantial homology to the sequence outlined above.

As noted above, the portable DNA sequences of the present invention may be synthetically created, by hand or with automated apparatus. It is believed that the means for synthetic creation of these polynucleotide sequences are generally known to one of ordinary skill in the art, particularly in light of the teachings contained herein. As examples of the current state of the art relating tc polynucleotide synthesis, one is directed to Maniatis et al., Molecular Cloning--A Laboratory Manual, Cold Spring Harbor Laboratory (1984), and Horvath et al. An Automated DNA Synthesizer Employing Deoxynucleoside 3'-Phosohoramidites, Methods in Enzymology 154:313-326, 1987, hereby incorporated by reference.

Additionally, the portable DNA sequence may be a fragment of a natural sequence, i.e., a fragment of a polynucleotide which occurred in nature and which has been cloned and expressed for the first time by the present inventors. In one embodiment, the portable DNA sequence is a restriction fragment isolated from a genomic library. In this preferred embodiment, the genomic library is created from the bacterium Vitreoscilla. In other alternative embodiments, the portable DNA sequence is isolated from other genomic and cDNA libraries.

While it is envisioned that the portable DNA sequences of this invention may desirably be inserted directly into the host chromosome, the present invention also provides a series of vectors, each containing at least one of the portable DNA sequences described herein. It is contemplated that additional copies of the portable DNA sequence may be included in a single vector to increase a host cell's ability to produce large quantities of the desired oxygen-binding protein. It is also envisioned that other desirable DNA sequences may also be included in the vectors of this invention. Further, the invention may be practiced through the use of multiple vectors, with additional copies of at least one of the portable DNA sequences of this invention and perhaps other desirable DNA sequences.

In addition, the cloning vectors within the scope of the present invention may contain supplemental nucleotide sequences preceding or subsequent to the portable promoter/regulator and/or DNA sequence. These supplemental sequences are those that will not adversely interfere with transcription of the portable promoter/regulator and/or any fused DNA sequence and will, in some instances, enhance transcription, translation, posttranslational processing, or the ability of the primary amino acid structure of the resultant gene product to assume an active form.

A preferred vector of the present invention is set forth in Figure 1. This vector, pUC19/pRED2, contains the preferred nucleotide sequence which codes for the amino acids set forth above. Vector pUC19/pRED2 cells are on deposit in the American Type Culture Collection ("ATCC") in Rockville, Maryland under Accession No. 67536.

A preferred nucleotide sequence encoding the Vitreoscilla hemoglobin protein and adjacent Vitreoscilla sequences described above is identified in Figure 1 as region A. The above nucleotide sequence reads counter-clockwise through region A of Figure 1. Plasmid pUC19/pRED2 may also contain supplemental nucleotide sequences preceding and subsequent to the preferred DNA sequence in region A, such as terminators, enhancers, attenuators and the like. For proteins to be exported from the intracellular space, at least one leader sequence and any other DNA sequences necessary or preferred for appropriate transcription and subsequent translation of the vector DNA may be included within the scope of this invention.

In a preferred embodiment, cloning vectors containing and capable of expressing the portable DNA sequence of the present invention contain various operational elements in addition to or instead of the promoter/regulator disclosed and claimed herein. These "operational elements" may include at least one promoter, at least one sequence that acts as expression regulator, and at least one terminator codon, at least one leader sequence, and any other DNA sequences necessary or preferred for appropriate transcription and subsequent translation of the vector DNA.

Additional embodiments of the present invention are envisioned as employing other known or currently undiscovered vectors which would contain one or more of the portable DNA sequences described herein. In particular, it is preferred that these vectors have some or all of the following characteristics: (1) possess a minimal number of host-organism sequences; (2) be stable in the desired host; (3) be capable of

being present in a high copy number in the desired host; (4) possess a regulatable promoter; and (5) have at least one DNA sequence coding for a selectable trait present on a portion of the plasmid separate from that where the portable DNA sequence will be inserted. Alteration of vectors to meet the above criteria are easily performed by those of ordinary skill in the art in light of the available literature and the teachings herein. It is to be understood that additional cloning vectors may now exist or will be discovered which have the above-identified properties and are therefore suitable for use in the present invention and these vectors are also contemplated as being within the scope of this invention.

As set forth in Example 1, an E. coli vector system is a preferred embodiment. Various cloning vehicles are required for the range of host cells and organisms suitable for insertion of the portable DNA sequences of the present invention, as set forth below. In light of the available literature, choice of such a cloning vehicle, if necessary, is within the ordinary skill in the art.

Additional bacterial hosts are suitable, including, without limitation: bacteria such as members of the genera Bacillus, Pseudomonas, Alcaligenes, Streptococcus, Lactobacillus, Methylophilus, Xanthomonas, Corynebacterium, Brevibacterium, Acetobacter, and Strepotomyces.

Examples of suitable eucaryotic host microorganisms would include fungi, yeasts such as Saccharomyces and Candida, and molds such as Aspergillus, Pennicillium, Trichederma and Cephalosporium (Acremonium).

It is envisioned that the scope of this invention would cover expression systems in eucaryotic microorganisms and host cultured cells derived from multicellular organisms, including animals, insects and plants, which are grown in the presence of oxygen. The promoter/regulator of the present invention is especially useful in a host which switches from low to very high expression activity upon reduction of dissolved oxygen concentration in the medium. Such expression systems need not be derived from Vitreoscilla.

Various vector systems will be suitable for these and other desirable hosts, including plasmids, viruses and bacteriophages. The following, noninclusive list of cloning vectors is believed to set forth vectors which can easily be altered to meet the above criteria and are therefore preferred for use in the present invention. Such alterations are easily performed by those of ordinary skill in the art in light of the available literature and the teaching herein.

For example, many selectable cloning vectors have been characterized for use in E. coli, including pUC8, pUC9, pBR322, pGW7, placI^{q}, and pDP8, Maniatis et al., supra. A bifunctional vector that replicates in E. coli and can also be used in Streptomyces is pKC462a. Suitable vectors for use in Bacillus include: pUB110, pSA0501, pSA2100, pBD6, pBD8, and pT127, Ganesan and Hock, eds., Genetics and Biotechnology of Bacilli, Academic Press 1984. In Pseudomonas, RSF1010, Pms149, pXT209, and RK2 are suitable; some of these vectors are useful in a wide range of gram-negative bacteria including Agrobacterium and Xanthomonas. For Saccharomyces, it is possible to use YEp24, YIp5, and YRp17, Botstein

and Davis, Molecular Biology of the Yeast Saccharomyces (Strathern et al., eds), Cold Spring Harbor Laboratory, 1982. In mammalian systems retrovirus vectors such as those derived from SV40 are typically used.

Synthesis and/or isolation of necessary and desired component parts of cloning vectors, and their assembly is believed to be within the duties and tasks performed by those with ordinary skill in the art and, as such, are capable of being performed without undue experimentation.

In construction of the cloning vectors of the present invention, it should additionally be noted that multiple copies of the promoter/regulator with any fused gene sequences and/or of the portable DNA sequence coding for the oxygen-binding protein and its attendant operational elements as necessary may be inserted into each vector. In such an embodiment, the host organism would produce greater amounts per vector of the desired oxygen-binding protein. The number of multiple copies of the DNA sequence which may be inserted into the vector is limited only by the ability of the resultant vector, due to its size, to be transferred into and replicated and expressed in an appropriate host.

Additionally, it is preferred that the cloning vector contain a selectable marker, such as a drug resistance marker or other marker which causes expression of a selectable trait by the host. In a particularly preferred embodiment of the present invention, the gene for ampicillin resistance is included in vector pUC19/pRED2. Such a drug resistance or other selectable marker is intended in part to facilitate in the selection of transformants.

Additionally, the presence of such a selectable marker on the cloning vector may be of use in keeping contaminating microorganisms from multiplying in the culture medium. In this embodiment, such a pure culture of the transformed host organisms would be obtained by culturing the organisms under conditions which require the induced phenotype for survival.

It is noted that the portable DNA sequence of the present invention may themselves be used as a selectable marker, in that they provide enhanced growth characteristics in low oxygen circumstances, and also engender an easily visible reddish tint in the host cells.

The promoter/regulators of this invention are capable of controlling expression of proteins or, thereby, of controlling synthesis of metabolites normally made by a cell, or of natural or unnatural metabolites and proteins expressed in a cell via genetic manipulation. This would include heterologous proteins--either intracellular or extracellular--as well as biopolymers such as polysaccharide materials, simpler metabolites such as amino acids and nucleotides, antibiotics and other chemicals produced by living cells or cellular biocatalysts.

The oxygen-binding proteins of the present invention, prepared by the recombinant-DNA methods set forth herein, will enable increased research into the growth of organisms in oxygen-poor environments. In addition, the oxygen-binding proteins of the present invention are useful in enhancing oxygen supply to cells or in other oxygen-utilizing processes (Adlercreutz et al., Biocatalyst in Organic Synthesis, Symposium of the Working Party on Immobilized Biocatalysts of the European Federation

of Biotechnology, Abstracts, p.18, 1985), and for binding and separating oxygen from other fluids or gases (Bonaventura et al., Underwater Life Support Based on Immobilized Oxygen Carriers, Applied Biochemistry and Biotechnology 9:65-80, 1984). Furthermore, the oxygen-binding proteins of this invention are capable of increasing production of cells, or of proteins or metabolites normally made by a cell, or of natural or unnatural metabolites and proteins expressed in a cell via genetic manipulation. This would, as described above, include heterologous proteins, biopolymers, simpler metabolites, antibiotics, and other chemicals produced by living cells or cellular biocatalysts.

The protein products of this invention also have applications as diverse as enhancing certain oxygen-requiring steps in fermentation, enzymatic degradation, toxic chemical waste treatment, brewing and particular oxidative reactions and transformations such as steroid conversions.

This invention also relates to a recombinant-DNA method for the production of oxygen-binding proteins. Generally, this method includes:

(a) preparing a portable DNA sequence capable of directing a host cell or microorganism to produce a protein having oxygen-binding activity;

(b) transferring the portable DNA sequence directly into the host, or cloning the portable DNA sequence into a vector capable of being transferred into and replicating in a host cell or microorganism, such vector containing

operational elements for the portable DNA sequence;

(c) transferring the vector containing the portable DNA sequence and operational elements into a host cell or microorganism capable of expressing the oxygen-binding protein;

(d) culturing the host microorganism under conditions appropriate for replication and propagation of the vector and/or expression of the protein.

In this method, the portable DNA sequences are those synthetic or naturally-occurring polynucleotides described above. In a preferred embodiment, the portable DNA sequence codes for at least a portion of the Vitreoscilla hemoglobin protein are described above.

This invention also relates to a recombinant-DNA method for the use of these promoter/regulators. Generally, this method provides a process for subjecting the expression of a selected DNA sequence to external control under given environmental conditions which comprises the steps of:

(a) providing at least one selected isolated structural gene that is transcriptionally and/or translationally responsive to a Vitreoscilla hemoglobin promoter/regulator DNA sequence under the given environmental conditions; and

(b) operatively fusing the selected structural gene with said promoter/regulator DNA sequence.

It is envisioned that the portable DNA sequences may be inserted directly into the host chromosome, or alternatively may utilize a vector cloning system. The vectors contemplated as being useful in the present method are those described above. In a preferred embodiment, the cloning vector pUC19/pRED2 is used in the disclosed method.

A vector thus obtained may then be transferred into the appropriate host cell or organism. It is believed that any microorganism having the ability to take up exogenous DNA and express those genes and attendant operational elements may be chosen. Particular hosts which may be preferable for use in this invention include those described above. Methods for transfer of vectors into hosts are within the ordinary skill in the art. For ultimate expression in certain microorganisms such as yeast, it may be desirable that the cloning vector be first transferred into another microorganism such as Escherichia coli, where the vector would be allowed to replicate and from which the vector would be obtained and purified after amplification, and then transferred into the yeast for ultimate expression of the oxygen-binding protein.

The host cells or microorganisms are cultured under conditions appropriate for the expression of the oxygen-binding protein. These conditions are generally specific for the host organism, and are readily determined by one of ordinary skill in the art, in light of the published literature regarding the growth conditions for such organisms.

In one embodiment for expressing globins, conditions necessary for the regulation of the expression of the DNA sequence, dependent upon any operational elements inserted into or present in the vector, would be in effect at the transformation and culturing stages. The cells are grown to a high density in the presence of appropriate regulatory conditions which inhibit the expression of the DNA sequence. When optimal cell density is approached, the environmental conditions are altered to those appropriate for the expression of the portable DNA sequence. It is thus contemplated that the production of a cloned protein will occur in a time span subsequent to the growth of the host cells to near optimal density, and that the resultant cloned protein product would be harvested, if desired, at some time after the regulatory conditions necessary for its expression were induced.

Where the operational elements used are in the promoter/regulator sequence of this invention, these conditions are as follows. The cells are grown to a high density in the presence of appropriate levels of oxygen which inhibit the expression of the DNA sequence. When optimal cell density is approached, the environmental oxygen level is altered to a lower value appropriate for the expression of the portable DNA sequence. Levels from less than about 1% oxygen-saturation to an oxygen saturated solution are within the scope of this invention. It is thus contemplated that the production of any desired fused product will occur in a time span subsequent to the growth of the host cells to near optimal density, and that the resultant product would be harvested, if desired, at some time after the oxygen level necessary for its expression were reached.

If harvesting of the oxygen-binding protein products of the present invention is desired, it may be done prior or subsequent to purification and prior or subsequent to assumption of an active structure.

It is currently believed that some percentage of the oxygen-binding proteins of the present invention will assume their proper, active structure upon expression in the host cell or organism. If desired, the oxygen-binding protein may be transported across a cell membrane. This will generally occur if DNA coding for an appropriate leader sequence has been linked to the DNA coding for the recombinant protein. The structures of numerous signal peptides have been published. It is envisioned that these leader sequences, included in or added to at least some portion of the portable DNA as necessary, will direct intracellular production of a fusion protein which will be transported through the cell membrane and will have the leader sequence cleaved upon release from the cell.

Additional uses of the oxygen-binding proteins of the present invention are envisioned. The purified proteins and/or the whole cells and/or extracts of the cells of the present invention themselves may be used to bind to oxygen or proteins and thus could function somewhat as erythrocytes.

The present invention may also be used as a method for transporting and enhancing oxygen supply to cells or in other oxygen-utilizing processes by delivering the oxygen-binding proteins--isolated in lysates and crude cell preparations, purified from extracts, in synthetic sequences, or in whole cells containing the proteins--where desired. It is envisioned that the protein products of the present invention could

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valuably be added to media for culturing cells and thereby enhance the transport of oxygen.

It is also envisioned that the proteins of the present invention may be used for binding and separating of oxygen from fluids such as seawater and from other gases.

It is understood that application of the teachings of the present invention to a specific problem or environment will be within the capabilities of one having ordinary skill in the art in light of teachings contained herein. Examples of the products of the present invention and representative processes for their isolation, use and manufacture appear below.

INDUSTRIAL APPLICABILITY

The products and processes of the present invention find usefulness in a range of medical, laboratory and industrial applications. The invention provides metabolically engineered cells with enhanced growth characteristics for increasing production of various proteins or metabolites by those cells. The invention further provides a method for subjecting expression of a certain DNA sequence to external control under given environmental conditions. Also provided are recombinant-DNA fusion gene products, expression vectors, and nucleotide base sequences for the practice of the invention. The products and processes of the present invention find applications in a range of aerobic processes, such as manufacture of cloned proteins and synthesis of metabolites, chemical production by fermentation, enzymatic degradation, waste treatment, brewing and a range of oxidative reactions.

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EXAMPLES

EXAMPLE 1 - CLONING AND EXPRESSION OF HEMOGLOBIN FROM VITREOSCILLA IN ESCHERICHIA COLI.

Materials and Methods. Vitreoscilla sp. (Murray strain no. 389) was obtained from Dr. Webster (Department of Biology, Illinois institute of Technology, Chicago, Illinois 60616, USA), and grown in a medium containing 1.5% yeast extract, 1.5% peptone, and 0.02% sodium acetate (pH 8.0 with NaOH). This strain is also available from ATCC, accession number 13981.

E. coli JM101 were obtained from the laboratory of Dr. Simon (Division of Biology, California Institute of Technology, Pasadena, California 91125, USA), and grown in L broth containing 1% Bactotryptone, 0.5% yeast extract and 1% sodium chloride. This strain is also available from ATCC, accession number 33876.

Plasmid pUC19 (Yanisch-Perron et al., Improved M13 phage cloning vectors and host strains: nucleotide sequences of m13mp18 and pUC19 vectors, Gene 33:103-109, 1985) packaging kits were purchased from Pharmacia. All restriction enzymes, T4 polynucleotide kinase and T4 ligase were from New England Biolabs or Bethesda Research Laboratories. Calf intestine alkaline phosphatase was from Pharmacia. Mixed oligonucleotide probes were synthesized with an Applied Biosystems synthesizer. Kodak XAR5 x-ray film was used for autoradiography. Geneclean kits were purchased from Bio101. All other chemicals were of analytical grade.

Vitreoscilla genomic DNA was isolated according to the protocol of Silhavy et al., Experiments with gene fusions, Cold Spring Harbor Laboratory (1984),

specifically incorporated herein. HindIII-digested Vitreoscilla DNA was ligated into the phosphatased HindIII site of pUC19 and transformed into JM101. Recombinant colonies and plaques were transferred on nitrocellulose filters as described in Maniatis, et al., Molecular cloning--a laboratory manual, Cold Spring Harbor Laboratory (1982) and specifically incorporated herein. Rapid plasmid isolation from recombinant colonies were done according to Silhavy et al., supra. Digested fragments of plasmid DNA or fractions of genomic DNA were isolated from agarose gels using Geneclean kits. E. coli cells were transformed by the $CaCl_2$ method of Silhavy et al., supra. Plasmid uptake was induced by heat-shocking chilled competent cells at 37°C for 5 minutes.

For Southern hybridizations the reagents suggested in Dupont catalog No. NEF-976, Protocols for electrophoretic and capillary transfer of DNA and RNA, DNA and RNA hybridization, and DNA and RNA rehybridization (1985), specifically incorporated herein, were used, whereas for colony and plaque hybridizations those described in Maniatis et al., supra, were used. Filters were prehybridized at 45-50°C for 2-4 hours and hybridized at 30°C for 20-24 hours. 200 picomoles oligonucleotide kinased with 200 microCi (^{32}P)ATP (sp. act. 7000 Ci/mmol) were used as probe. Filters were washed in 2 X SSC, 0.1% SDS at room temperature (3 X 5 minutes) and at 46°C (for the C-terminal probe) and 50°C (for the N-terminal probe) prior to autoradiography.

SDS-polyacrylamide gel electrophoresis was done according to standard protocols, Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227:580-685, 1970, specifically incorporated herein, with a 12.5%

resolving gel. Protein in the gel was visualized by the silver staining method or Merril et al., Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins, Science 211:1437-1438, 1983.

Results. Three sets of mixed oligonucleotide probes were synthesized which had a predicted homology to two domains in the hemoglobin gene, one N-terminal and one C-terminal. A pUC19-HindIII library of Vitreoscilla DNA was test-plated on rich plates with ampicillin, X-gal and IPTG. More than 70% of the colonies were probable recombinants, as estimated by visual inspection. About 10,000 colonies were then screened. Three positives were identified. Because of the high density of colonies on the plate, these, along with their immediate clones from each group, were assayed by rapid isolation and HindIII digestion. One of these, pRED1, had three inserted fragments including a 2.2kb one. Subsequent digestion of this plasmid with various endonucleases and Southern hybridization of the resulting DNA bands did confirm that the 2.2kb band did indeed contain the entire hemoglobin gene, since no HindIII sites are expected to exist upstream or downstream of the regions spanned by the oligomeric probes.

The HindIII fragment from pRED1 that contained the hemoglobin structural gene was purified and reinserted by standard protocols into pUC19 in both orientations (pRED2 and pRED3). E. coli cells containing plasmids pRED1, pRED2, pRED3 and pUC19 as well as Vitreoscilla cells were grown to stationary phase and cell extracts were assayed on an SDS-polyacrylamide gel for the existence of the hemoglobin polypeptide. The hemoglobin was expressed as a major cellular protein in all recombinant cells.

Since both plasmids PRED2 and PRED3 express about equal amounts of this polypeptide, it is presently believed that the gene is probably expressed from its natural promoter in E. coli.

The restriction map of plasmid pRED2 is shown in Figure 1.

Determination of the sequence of the relevant region of the fragment isolated from the Vitreoscilla Ugenomic library was accomplished as follows:

The HindIII-SphI fragment from plasmid pRED2 which contains the structural gene and adjacent sequences was subcloned into pUC19 (purchased from Bethesda Research Labs) to obtain plasmid pRED4. An MluI site was identified, by restriction mapping the resulting plasmid, which breaks up the HindIII-SphI insert into two fragments which were individually sequenced using conventional protocols (Maxam and Gilbert, Sequencing end-labeled DNA with base-specific chemical cleavages, Methods in Enzymology 65:499-560, 1980; Iverson and Dervan, Adenine specific DNA chemical sequencing reaction, Nuclear Acids Research 15:7823-7830, 1987).

The nucleotide sequence of the important portion of the HindIII-SphI fragment includes a putative E. coli promoter, ribosome binding site, the complete VHb structural gene (start and stop codons are underlined) and a putative E.- coli transcription terminator (Khosla and Bailey, The Vitreoscilla hemoglobin gene: molecular cloning nucleotide sequence and genetic expression in Escherichia coli, Mol. & Gen. Genet., 214:158-161 (1988).

EXAMPLE 2 - GROWTH ENHANCEMENT IN E. COLI WITH pPRED2: SHAKE FLASK CULTURES.

In this Example, the growth behavior of E. coli cells producing active Vitreoscilla hemoglobin was compared to that of control strains grown under identical conditions. The following strains were studied: (1) JM101:pRED2; (2) JM101:pUC9; and (3) JM101. Plasmids pUC9 and pUC19 are essentially identical except for a difference in one restriction site unrelated to the insert or to any of the functional properties of the plasmid.

Experimental protocol: Cells were grown at 37°C in a complex medium containing 1% (W/V) bactotryptone, 0.5% (W/V) yeast extract, 0.5% (W/V) NaCl, 0.3% (W/V) K_2HPO_4 and 0.1% (W/V) KH_2PO_4 (pH 7.0). Plasmid-containing cells were grown in the presence of 100 mg/L ampicillin. In each case the shake-flask was inoculated with a 1% (V/V) dose of concentrated nutrient broth containing 430 g/L glucose, 5 g/L yeast extract, 110 g/L $(NH_4)_2SO_4$, 8 g/L $MgSO_4 \cdot 7H_2O$, 0.27 g/L $FeCl_3 \cdot 6H_2O$, 0.02 g/L $ZnCl_2 \cdot 4H_2O$, 0.02 g/L $CaCl_2 \cdot 2H_2O$, 0.02 g/L $NaMoO_4 \cdot 2H_2O$, 0.01 g/L $CuSO_4 \cdot 5H_2O$, 0.005 g/L H_3BO_{31} 0.1% (V/V) conc. HCl, 4.2 mg/L riboflavin, 54 mg/L pantothenic acid, 60 mg/L folic acid. This formulation has been successfully used on a previous occasion to grow stationary cells to a high density in a fedbatch mode. The cells were then allowed to grow further until stationary phase was reached again. Optical density was measured at 600 nm on a Bausch & Lomb Spectronic 21 spectrophotometer. Dry weights were measured by spinning 10 mL samples at 4°C, washing once with distilled water and subsequently drying the resuspended sample at 100°C overnight. The heme content of the cells was assayed according to the method of Lamba & Webster (Lamba & Webster, Effect of growth conditions on yield and heme content of Vitreoscilla, Journal of Bacteriology

142:169-173, 1980), and the hemoglobin activity was measured by the method of Webster & Liu (Webster and Liu, Reduced nicotinamide adenine dinucleotide cytochrome o reductase associated with cytochromic o purified from Vitreoscilla, Journal of Biological Chemistry, 249:4257-4260, 1974.).

Results. The growth properties, heme content and hemoglobin activity of the three strains are documented in the Table below.

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		JM101:pRED2	JM101:pUC9	Jm101
1.	OD_{600} before nutrient replenishment	0.937	0.737	0.945
2.	OD_{600}	1.230	0.880	0.985
3.	max. attained dry wt.	1.5g/L	0.85g/L	1g/L
4.	relative heme content	5.5	1	**
5.	relative hemoglobin activity	5	I	**
6.	specific growth rate*	0.04/h	0.01/h	0.009/h

*mean value following additional feeding of shake-flasks as described above

**not assayed

EXAMPLE 3 - GROWTH ENHANCEMENT OF E. COLI WITH pRED2.

A typical high-cell density fermentation is of a fedbatch type. The optimal rate of nutrient addition, and consequently the productivity, is ultimately limited by the rate at which cells can aerobically catabolize the carbon source without generating growth-inhibitory metabolites such as acetate and lactate (Zabriskie and Arcuri, Factors influencing productivity of fermentations employing recombinant microorganisms, Enzyme and Microbial Technology 8:706-717, 1986; Tsai et al, The effect of organic nitrogen and glucose on the productivity of recombinant insulin-like growth factor in high cell density Escherichia coli fermentations, Journal of Industrial Microbiology 2:181-187, 1987). In this experiment, we compare the growth properties of the

recombinant strain (JM101:pRED2) expressing Vitreoscilla hemoglobin with similar plasmid-containing (JM101:pUC9) and plasmid-free (JM101) strains under typical fed-batch fermentation conditions.

Materials and Methods:

Cells were grown in a New Brunswick Microferm fermentor at 37±0.5°C and a pH of 7±0.05 with an initial working volume of 2.5 L. A constant air-flow rate of 4.5 L/min and agitator speed of 300 rpm were maintained throughout each run. Silicone antifoam AF60 was used to control foaming. The batch medium and feed medium 1 listed in Table 2 in Tsai et al, supra were used. Growth following inoculation was in batch mode. After batch stationary phase was reached, continuous feeding was initiated using feed medium 1 at a flow rate of 10 mL/hr. For plasmid-containing cells, 100 mg/L ampicillin was used. In all cases, the dissolved oxygen (DO) levels remained fairly constant around 5% of air saturation for most of the run except during the early log phase and towards batch stationary phase.

Results:

The growth parameters measured for the three strains are listed below. Batch stationary phase refers to conditions before continuous feeding was started.

	JM101:pRED2	JM101:pUC9	JM101:pRED2
Batch log-phase growth rate (h^{-1})	0.95	0.73	0.95
Batch stationary-phase dry cell mass (g/L)	2.6	1.6	2.6
Fed-batch log-phase growth rate (h^{-1})	0.056	0.033	0.066
Final dry cell mass (g/L)	5.8	2.8	5.9

Further, the respiratory behavior of JM101:pRED2 was improved compared to the control strains at low DO levels, as observed in a Gilson respirometer.

Conclusion:
Cells containing Vitreoscilla hemoglobin grow faster and to higher densities than comparable plasmid-containing controls.

EXAMPLE 4 - EXPRESSION OF VITREOSCILLA HEMOGLOBIN (VHb) IN E. COLI UNDER THE REGULATION OF OTHER PROMOTERS.

In Examples 1, 2, and 3 above, the expression of hemoglobin is under the regulation of its native oxygen-regulated promoter. Hence, it is not possible to modulate independently the dissolved oxygen concentration (DO) and the intracellular VHb level. In order to overcome this, the inventors attempted to express this protein under the control of other regulatable promoters which are functional in E. coli, such as trp (Russell and Bennett, Construction and analysis of in vitro activity of E. coli promoter hybrids and promoter mutants that alter

the -35 to -10 spacing Gene 20:231-243, 1982) and tac (deBoer et al, The tac promoter: a functional hybrid derived from the trp and lac promoters Proc. Natl. Acad. Sci. USA 80:21-25, 1983). Materials and Methods:

Plasmid PRED4 was linearized with HindIII and treated with exonuclease Ba131 to generate 5' end deletions in the HindIII-SphI VHb fragment (Maniatis et al, supra). After digestion with SphI, the resulting VHb fragments were cloned into HindIII-SphI digested pUC19. The positions of the deleted end-points were identified by sequencing.

trp and tac promoters and the chloramphenicol acetyl transferase gene (CAT) were purchased from Pharmacia, Inc. Oligonucleotides were synthesized at California Institute of Technology using an Applied Biosystems DNA synthesizer.

All DNA enzymes were obtained from vendors.

The functional assay for the VHb gene product is as described in Webster and Liu, supra.

Cells were pelleted at 4°C and resuspended in 100 mM Tris (pH 7.5), 50 mM NaCl. This cell suspension was sonicated at 75 W for 3 min. on ice. After spinning in at 12,000 g for 10 min., the supernatant was collected and assayed for VHb. Total protein content was estimated using the Bradford assay kit from BioRad Inc. Vhb activity is reported as delta-$A_{419-436}$/mg total protein.

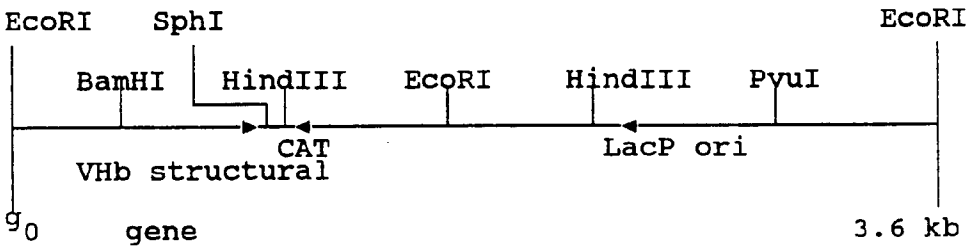
Results:

One of the deletions, pRED302, mapped 2 base-pairs upstream of the ATG start codon for the VHb structural gene. This deletion was used for further work. The EcoRI/BamHI trp promoter cartridge was cloned upstream of the truncated VHb fragment. The following ribosome binding site was synthesized:

5' GATCCCGGGTCTAGAGGA 3'
GGCCCAGATCTCCT

and inserted between the BamHI and nuclease-blunted XbaI sites to give rise to a trp promoter-controlled VHb expression system. The CAT gene (Alton and Vapnek, Nucleotide sequence analysis of the chloramphenicol resistance transposon Tn9, Nature 282:864-869, 1979) was inserted downstream and under the control of the lac promoter available on this PUC19-based plasmid. This gene product can be conveniently assayed (Neumann et al, Novel rapid assay for chloramphenicol acetyltransferase gene expression, BioTechniques 5:444-447, 1987) and hence serves as a useful reporter. Finally, the ß-lactamase gene on this PUC19-based plasmid was deleted by digestion and religation with PvuI. The purpose of this step is to eliminate the presence of a plasmid-encoded periplasmic protein. The plasmid thus obtained was called pHbCAT and was transformed into JM101. As a control, the CAT gene was cloned downstream and under the control of the lac promoter in pUC19. The ß-lactamase gene was identically deleted. This plasmid was called pCAT. The restriction maps and the anticipated sequence of relevant regions of these two plasmids are shown below.

pHbCAT (3.6 kb)



EcoRI

5' GAATT CCCCT GTTGA CAATT AATCA TCGAA CTAGT TAACT
AGTAC

BamHI

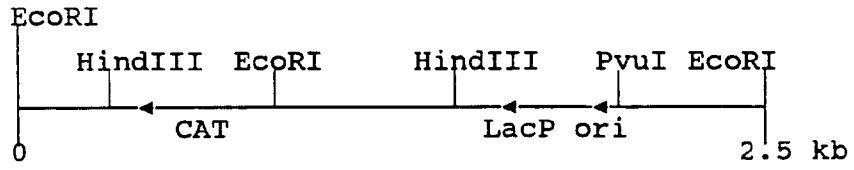
GCAGC TTGGC TGCAG GTCGA CGGAT CCCGG G CTA CAGGA
AGTCT

Start codon of VHb

CATGT TAGAC...(same as in Example 2 up to SphI site)

The sequence of the region spanning between EcoRI and the start of the VHb structural gene is shown above. It includes the trp promoter and a synthetic ribosome binding site.

pCAT (2.5 kb)



The effect of tryptophan (repressor) and indole acrylic acid (gratuitous inducer) on VHb levels in JM101/pHbCAT are shown in the Table below. In these experiments, cells were grown to mid-log in minimal

medium containing 3 g/L glycerol, 3 g/L Casamino acids, and the appropriate amount of indole-acrylic aced or tryptophan.

Host: Plasmid:	Tryptophan (mg/L)	Indole-acrylic acid (mg/L)	Specific Hb Activity*
JM101:pCAT	--	--	3.4×10^{-3}
JM101:phbCAT	20	--	6.2×10^{-3}
"	4	--	18.3×10^{-3}
"	--	--	31.5×10^{-3}
"	--	1	29.8×10^{-3}
"	--	2.5	36.5×10^{-3}
"	--	5	47.0×10^{-3}
"	--	10	36.6×10^{-3}

Note *(delta-$A_{419-436}$/mg total soluble protein)

To express the VHb gene under the control of the tac promoter, an expression plasmid plasmid was made using a HindIII-BamHI tac promoter cartridge, the BamHI/SphI fragment from pHbCAT, and the HindIII-SphI digested fragment of the vector pBR322 (Bolivar et al, Construction and characterization of new cloning vehicles. II. A multipurpose cloning system Gene 2:95-113, 1977).

With this construct (pINT1), the level of redness of cells correlated well with varying amounts of the gratuitous inducer IPTG, indicating that the gene product synthesis was under the control of tac. The advantages of this expression system are:

a. Higher expression of vHb, and

b. Ability to use complex medium for growth.

EXAMPLE 5 - GROWTH OF E. COLI - EXPRESSING VHb UNDER THE REGULATION OF OTHER PROMOTERS.

The aim of this experiment was to demonstrate the growth effects of VHb on E. coli. In these cases, VHb is expressed using promoters different from the native VHb oxygen-regulated promoter. The strains:plasmids used are:

1. HB101:pBR322 (pBR322 from BRL)

2. JM101:pINT1 (pINT1 discussed in Example 5).

The two hosts have nearly identical genotypes, the major difference being the presence of an F' factor in JM101 which harbors the $lacI^8$ gene. This gene is necessary to keep a strong promoter like tac under control.

The following media recipes shall be henceforth referred to in the appropriate annotated form:

1X LB: 10 g/L Bactotryptone, 5 g/L Yeast Extract, 5 g/L NaCl, 3 g/L K_2HPO_4, 1 g/L KH_2PO_4, 100 mg/L Ampicillin

2X LB: 20 g/L Bactotryptone, 10 g/L Yeast Extract, 5 g/L NaCl, 3 g/L K_2HPO_4, 1 g/L KH_2PO_4, 100 mg/L Ampicillin

5X LB: 50 g/L Bactotryptone, 25 g/L Yeast Extract, 5 g/L NaCl, 3 g/L K_2HPO_4, 1 g/L KH_2PO_4, 100 mg/L Ampicillin.

The experiment was conducted as follows. Single colonies of the two strains listed above were inoculated into 5 mL 1X LB in a culture tube and grown overnight at 37°C.

0.5mL of the appropriate inoculum was transferred into 250mL culture flasks containing 50 mL medium as follows:

1) HB101:pBR322 : 2X LB
2) HB101:pBR322 : 5X LB
3) JM101:pINT1 : 2X LB
4) JM101:pINT1 : 2X LB + 0.1 mM IPTG
5) JM101:pINT1 : 2X LB# 0.5 mM IPTG
6) JM101:pINT1 : 5X LB
7) JM101:pINT1 : 5X LB# 0.1 mM IPTG
8) JM101:pINT1 : 5X LB# 0.5 mM IPTG

Cells were then grown for 24 h at 37°C in a New Brunswick G24 Environmental Incubator Shaker with the shaker speed adjusted to medium setting. At the end of the experiment, the OD_{600} was measured in a Spectronics 21 spectrophotometer by diluting the culture 10-fold in 1% NaCl. The data are listed below.

Host/Plasmid	LB conc.	IPTC conc. (mM)	Final OD_{600}
HB101:pBR322	2X	0	3.00
JM101:pINT1	2X	0	3.03
JM101:pINT1	2X	0.1	2.91
JM101:pINT1	2X	0.5	3.00
HB101:pBR322	5X	0	2.73
JM101:pINT1	5X	0	3.26
JM101:pINT1	5X	0.1	3.40
JM101:PINT1	5X	0.5	3.15

From this data, the following conclusions may be drawn:

1. In all cases involving 2X LB, the cells grew to approximately the same density. This density is roughly twice that obtained routinely in IX LB under similar growth conditions and indicates exhaustion of available nutrient. In other words, cells have entered stationary phase of growth due to nutrient limitation.

2. It has been demonstrated (Tsai et al., supra) that cells grown in excess nutrient eventually attain an oxygen-limited growth condition due to which they generate inhibitory metabolics such as acetate. Eventually, this leads to cessation of growth, even if more nutrient is supplied. The results of all 5X experiments are indicative of such an occurrence. In other words, oxygen limitation has arisen eventually, causing the culture to reach stationary phase.

3. Hence, it may be argued that under O_2-limited growth, the presence of the hemoglobin gene enhances

the growth characteristics of E. coli. This result is similar to that in Examples 3 and 4, with the difference being that here VHb expression is not regulated by DO levels.

4. It appears that under the given growth conditions, there exists an optimal level of VHb expression that maximizes the growth enhancement effect. Such an optimum may be a function of specific growth properties of each cell line and/or plasmid construct as well as of the environmental conditions of growth. The optimum may thus have to be determined for different applications of this technology on a case-by-case basis; however, such determination does not require undue experimentation.

EXAMPLE 6 - EFFECT OF VHb PRESENCE ON THE SYNTHESIS OF ANOTHER CLONED PROTEIN IN E. COLI.

The aim of this experiment was to demonstrate the effect of the VHb gene on the synthesis of a model cloned gene product. This is an important application of the technology, since a wide variety of gene products are produced commercially via recombinant DNA technology. A typical process of this kind involves a high cell density fed-batch fermentation. The productivity of such processes is ultimately limited by insufficient oxygen availability.

The following hosts/plasmids were used in this example:

1. JM101:PCAT
2. JM101:PHbCAT.

The construction of these plasmids is described in Example 5.

The following media compositions were used:

LB: 10 g/L Bactotryptone, 5 g/L Yeast Extract, 5 g/L NaCl, 3 g/L K_2HPO_{41} 1 g/L KH_2PO_4, 30 mg/L Chloramphenicol

10X feed: 100 g/L Bactotryptone, 100 g/L Yeast Extract, 150 mg/L Chloramphenicol.

The experiment was conducted as folLows:

Single colonies of the two strains were inoculated into 5 ml LB in a culture tube and grown overnight. 1 mL of the inoculum was transferred into 100 mL fresh LB and the growth curve was followed. As cells approached the end of log phase, a 1 mL pulse of 10X feed was added and the growth burst was followed. A second pulse was similarly added. At the end of this growth phase, a pulse of 1 mL 10X feed containing 100 mM IPTG was added to induce the expression of the CAT gene. One hour later, a sample was withdrawn for monitoring CAT activity. The results of the experiment are shown below.

	JM101:pCAT	JM101:pHbCAT
1) Klett before IPTG pulse	670	700
2) Total soluble protein (mg/ml culture broth)	0.31	0.435
3) CAT activity (units/mg soluble protein)	$1.39x10^4$	$2.67x10^4$
4) CAT activity (units/ml culture broth)		

From the above data, the following conclusions may be drawn.

1. The presence of VHb enhances the synthesis of a cloned gene product, even at low levels of VHb industrion.

2. Besides increasing the amount of cloned gene product per unit volume of culture, the presence of VHb may also enhance the specific activity (activity per unit amount of totally soluble protein) of the cloned gene product.

EXAMPLE 7 - OXYGEN-DEPENDENT REGULATION OF EXPRESSION OF VHb IN E. COLI BY NATIVE VITREOSCILLA HEMOGLOBIN UPSTREAM SEQUENCES.

The aims of this experiment were as follows:

1. To demonstrate that VHb gene expression in E. coli increases in response to decreasing oxygen levels in the medium.

2. To establish transcriptional-level regulation of gene expression.

3. To determine the sensitivity of this oxygen-dependent genetic switch in response to changes in dissolved oxygen concentrations.

Materials and Methods:

The HindIII-SphI fragment containing the VHb gene and flanking sequences was cloned into the corresponding sites of the vector pBR322, thereby creating the plasmid pOX1. This was then transformed into the E. coli hold, HB101. The fermentation was conducted in a New Brunswick Bioflo II fermentor with a 2.5 L working volume using LB (10 g/L Bactotryptone, 5 g/L yeast extract, 5 g/L NaCl, 3 g/L K_2H-PO_4, 1 g/L KH_2PO_4) plus 8 mg/L silicone antifoam as medium at 37°C, pH 7.0 with a constant agitation speed of 300

rpm. All other methods involve conventional protocols (Maniatis, et al., supra).

Cells were grown to an OD_{600} ≈ 0.25 with DO maintained greater than 50% air saturation at all times. At this point, the air supply was gradually reduced so that the DO fell to about 1% air saturation in an almost linear manner over a period of 45 min. (i.e., a time scale long enough for gene induction, yet within approximately one generation time of E. coli). Samples were intermittently taken and analyzed for VHb mRNA and protein levels. Later, nitrogen was sparged in the vessel to study the induction of the VHb promoter under strictly anaerobic conditions.

Results:

1. The level of VHb mRNA increased about ten-fold as DO dropped from 70% to 1% air saturation.

2. There was a corresponding increase in VHb activity. A lag was noticed between increase in VHb mRNA level and increase in the quantity of active VHb. This may occur because of the requirement of additional heme biosynthesis in the host cell in order to produce active VHb.

3. The VHb promoter was switched on to significant levels only below 40% air saturation and attains maximum induction levels below 5% air saturation.

4. The promoter switches off under strictly anaerobic conditions, indicating the importance of a basal level of aerobicity in the environment for maximal gene expression.

EXAMPLE 8 - HETEROLOGOUS EXPRESSION OF VHb IN A EUCARYOTIC SACCHAROMYCES CEREVISIAE HOST.

In this experiment, we attempted to express the VHb gene in a model eucaryote, Saccharomyces cerevisiae. The vector used was pBM 150 (Johnston and Davis, Sequences that regulate the divergent GAL1-GAL10 promoter in Saccharomyces cerevisiae, Molecular and Cellular Biology 4:1440-1448, 1984) and the host strain was D603 (ade^-, ura^-, met^-, lys^-, reg 1), Srienc et al., Effect of ARS1 mutations on chromosome stability in Saccharomyces cerevisiae, Molecular and Cellular Biology 5:1676-1684, 1985. The truncated VHb structural gene referred to in Example 4 was cloned downstream of the GAL10 promoter to create plasmid pYRED1.

Results:

The recombinant strain D603:pYRED1 was significantly redder than the control D603:pBM150 when grown in the presence of galactose (2% peptone, 1% yeast extract, 2% galactose). Inocula were grown in minimal galactose medium. Significant hemoglobin activity was determined in sonicates from D603:pYRED1 compared to D603:pBM150 controls based on the difference spectrum hemoglobin analysis referenced in Example 2.

EXAMPLE 9 - GROWTH ENHANCEMENT OF SACCHAROMYCES CEREVISIAE CELLS EXPRESSING VHb.

In this example, the effect of VHb expression on the growth of the yeast Saccharomyces cerevisiae was studied. The VHb gene was cloned into a yeast expression plasmid, AAH5, that is stably maintained as an extrachromosomal plasmid in yeast cells.

Materials and Methods:

Plasmid pEX-2 was constructed as follows. The BamHI/SphI fragment described in Example 4 was cloned by blunt-end ligation into the HindIII site of the

yeast expression vector AAH5 (Ammerer, Expression of genes in yeast using the ADC1 promoter, Methods in Enzymology 101:192-201, 1983). AAH5 contains the selectable yeast marker Leu 2, the 2 micron circle origin of replication, and a unique HindIII site flanked by the transcriptional promoter and terminator regions of the yeast alcohol dehydrogenase-1 (ADH-1) gene. The ADH-1 promoter will support high levels of transcription of any sequence cloned into the HindIII site. The ADH-1 gene is constitutively expressed in yeast.

S. cerevisiae strain 488-0 (leu2, ura3, his 1-7) was transformed with plasmids AAH5 and PEX-2 by the rapid colony transformation procedure (Keszenman-Pereyra and Heida, A colony procedure for transformation of Saccharomyces cerevisiae, Curr. Genet. 13:21-23, 1988), and plated on synthetic dextrose (SD) medium (Rose, Isolation of genes by complementation in yeast, Methods in Enzymology, 152: 481-504, 1987) without leucine. A representative clonal cell line from each transformation was established after colony purification of a primary transformant.

For the growth studies, single yeast colonies were inoculated into 2 mL of SD -leu (+leu for 488-0) and cultured for 24 hr at 260 rpm at 30°C in a Labline Model 3258 Orbit Enviro-shaker. 0.5 mL of this inoculum was added to 50 mL of the same medium in a 250 mL flask and cultured at 260 rpm at 30°C. Cell growth was measured by turbidity (A600nm) using a Perkin-Elmer Lambda 4A Spectrophotometer. When the glucose level of the culture medium dropped below 2.0 mM, the cultures were pulsed with 1/40 volume of a concentrated medium containing 20 x SD (40% glucose, 13.3% Difco yeast nitrogen base without amino acids, and 1.6 mg/mL of all the amino acids except leucine.

For strain 488-0, 1.6 mg/mL leucine was included in the pulse medium). Glucose concentration was estimated using Ames Glucostix test strips.

Results:

A comparison of the growth curves Of strains 488-0, 488-0:AAH5, and 488-0:pEX-2 grown under the above conditions revealed the following:

1. All three strains grew at an equivalent rate during the logarithmic stage of growth.

2. The VHb-containing strain 488-0:pEX-2 grew to a final optical density of 13.0, while strains 488-0:AAH5 and 488-0 grew to optical densities of only 10.0 and 9.5, respectively. This represents a 26.0% increase in final cell density between a strain carrying the VHb gene on a plasmid (488-0:pEX-2) compared to a strain containing the identical plasmid without the VHb gene (488-0:AAH5). In addition, this represents a 32.6% increase in the final cell density of 488-0:pEX-2 over the strain containing no AAH5-derived plasmid (488-0).

EXAMPLE 10 - GROWTH ENHANCEMENT DUE TO EXPRESSION OF VHb IN E. COLI FROM A CHROMOSOMICALLY INTEGRATED GENE.

In this example, the tac-VHb gene fusion, discussed in Example 4, was integrated into the chromosome of E. coli MG1655 (obtained from Cold Spring Harbor Laboratory, NY).

Materials and Methods:

A defective Tn10 transposon (Foster, et al., Three Tn10-associated excision events: Relationship to transposition and role of direct and inverted repeats, Cell, 23:215-227, 1981) was constructed as follows. A kanamycin resistance gene (Pharmacia

Inc.) was cloned into the SalI site of PINT1 (Example 5). The EcoRI/EagI fragment from the resulting plasmid, which contains the entire tac-VHb fusion and Kan^R gene, was cloned between the inverted repeats (bases 1-66 on the right end and bases 9234-9300 on the left end) of a Tn10 derivative which lacks the transposase gene (obtained from Cold Spring Harbor Laboratory, NY). The resulting element, Tn10dKan-tac-VHb, was cloned into a multicopy plasmid containing a tac-Tn10 rightward transposase (obtained from Cold Spring Harbor Laboratory, NY). Transposition was induced with 0.5 mM IPTG for 4 hr, following which cells were plated on lactose-MacConkey-Kan plates. Lac-mutants were selected and the transposon-induced mutation was induced into E. coli MG1655 using Pi phage (Silhavy et al., supra). One of the resulting Lac-colonies, which was further purified and checked for Lac^-, Kan^R, Amp^S, VHb^+ (IPTG inducible, as confirmed by assay described in Example 3), was designated GR013. Comparison of growth properties of strains MG1655 and GR013 in 2X LB (described in Example 6) containing 1 mM IPTG, followed by addition of a concentrated feed (25% Bactotryptone, 12.5% yeast extract), showed an increase in final cell densities (final cell densities: OD_{600} = 16.8 for MG1655, OD_{600} = 18.1 for GR013).

EXAMPLE 11 - CONSTRUCTION OF AN EXPRESSION VECTOR FOR CULTURED MAMMALIAN CELLS

The eukaryotic expression vector pMSG (Pharmacia LKB Biotechnology, Piscataway, NJ) was propagated in *E. coli* HB101. *E. coli* HB101 was transformed with pMSG using a standard $CaCl_2$ protocol (1). After culturing transformants overnight, pMSG plasmid DNA was isolated by standard miniprep technique (1). The authenticity of this plasmid was confirmed by

performing several restriction digestions of the original and the HB101-derived pMSG plasmid DNA. A frozen stock of HB101-pRED302 (2) was plated out on a agar plate. The following day, a 10 ml overnight culture was inoculated from a single colony. Miniprep plasmid isolation was carried out on this overnight culture for pRED302 isolation. Plasmid pRED302 was digested with Xbal and Sspl (Boerhringer Mannheim, Indianapolis, IN) and the digested sample was run on a 0.7% agarose gel. This resulted in 3 fragments of approximate sizes of 2.5 kb, 0.56 kb and 0.54 kb. Test digestions were carried out with the two smaller fragments using the restriction enzymes Mlul and Bsu361 since these sites are present in the VHb structural gene. Electrophoresis of these digested samples on a 2% agarose gel confirmed that the lower band contains the VHb structural gene. The lower band was eluted and purified from the gel using the GeneClean Kit (Bio 101, La Jolla, CA) resuspended in 30 ml TE (pH 8.0) and stored at -20°C. Part of this purified fragment was used for construction of pMSG-VHb and remaining sample was frozen down at -20°C for use in Southern hybridization experiments. The construction of pMSG-VHb was done by utilizing the multicloning site of pMSG. pMSG was sequentially digested with Sma1 and Nhe1 with an intermediate phenol:chloroform extraction and ethanol precipitation step. The digested vector was run on a 0.7% agarose gel and the larger fragment was purified using a GeneClean Kit, resuspended in 20 ml TE (pH 8.0) and stored at -20°C. An overnight blunt-sticky end ligation reaction was carried out at 8-15°C for cloning the Xba1/Sspl VHb fragment into the Nhe1/Sma1 digested pMSG. This vector was named pMSG-VHb. The following day, competent HB101 were transformed separately with no plasmid, pMSG and pMSG-VHb respectively and spread on LB-ampicillin plates.

Nine colonies were picked from pMSG-VHb plate as potential transformants for further analysis. Minipreps were carried out on overnight cultures of all these potential transformants for pMSG-VHb plasmid DNA isolation. Vectors pMSG and pMSG-VHb were digested with the differed restriction enzymes BamH1, Hing111, Sal1 and M1u1 for verifying the authenticity of the new construct. When run on a 0.7% agarose gel, those samples confirmed the presence of the VHb insert in the multicloning site of pMSG. A maxiprep was carried out using two 100 ml cultures of one of the pMSG-VHb transformants for large-scale plasmid DNA isolation. The DNA was extracted with phenol:chloroform, ethanol precipitated, the pellet resuspended in 1 ml TE (pH 8.0) and stored at -20°C until further use in CHO cell transfection.

EXAMPLE 12 - TRANSFECTION OF CHO CELL WITH AN EXPRESSION VECTOR

A Chinese hamster ovary (CHO) cell line producing tPA (ATCC 9606) was obtained from ATCC (Bethesda, Maryland). These cells were grown routinely in a non-selection medium containing DMEM (high glucose) (GIBCO, Grand Island, NY) supplemented with 1X penicillin-streptomycin-glutamine (Irvine Scientific, Irvine, CA) solution and 5% dialyzed FBS (GIBCO, Grand Island, NY) in a 5% CO_2 humidified incubator at 37°C. Tissue culture dishes (100 & 20mm) were used in all the experiments. The selection medium for transfected cells contains 25 mg/1 mycophenolic acid (Grand Island, NY), 1 X HAT (Gibco, Grand Island, NY) and 250 mg/1 xanthine (Sigma, St. Louis, MO in addition to the non-selections medium components. During regular cultures, cells were passaged every 2-3 days upon semi-confluency. Total cell counts and cell size distribution were monitored by the Coulter

counter. Cell viability was determined using the trypan blue exclusion method.

The CHO cells producing tPA were transfected with pMSG-VHb using the standard calcium phosphate procedure described in Maniatis (1). Briefly, one 100 x 200 mm tissue culture dish with 10 ml non-selection medium was inoculated with 1 x 10^6 cells 24 hours prior to transfection. Twenty mg pMSG-VHb DNA was digested overnight with EcoR1 at 37°C. This linearized vector DNA was subjected to phenol:chloroform extraction and ethanol precipitation and the resulting pellet was resuspended in an appropriate volume of 0.1 X TE (pH 8.0). This was combined with 20 mg of carrier DNA and the calcium phosphate-DNA precipitate was formed according to the standard protocol. 1 ml of this precipitate was added to the 100 mm dish containing cells. These cells were incubated for 24 hours at 37°C upon which the medium was aspirated and fresh non-selection medium added in order to facilitate the expression of XGPRT. Cells were then diluted 1:8 in selection medium and allowed to grow in this medium. The medium was replaced every two days and single colonies began to appear in about two weeks. About 40 colonies resistant to selection pressure were picked using cotton tips wetted with trypsin and transferred to 24-well tissue culture plates. These colonies were expanded to 60 and 100 mm dishes upon reaching confluency in the wells. All except 3 clones were frozen down at -70°C. The three clones (clones 4, 19 and 30) were gradually expanded to 100 mm dish cultures for further studies.

EXAMPLE 13 - VALIDATION OF CLONING OF THE VHb GENE IN ENGINEERED OR RECOMBINANT CHO CELLS

Genomic DNA was isolated from various clones using standard protocos from Maniatis et al. (1). 5 ng DNA each of the parental CHO-tPA and three VHb-CHO clones were digested overnight with EcoR1 at 37°C. Agarose gel electrophoresis of these digested DNAs was carried out using 0.5 X TBE for six hours at 60 volts. The DNA was then transferred overnight to Immobilon-S membrance (Millipore, Bedford, MA) using a capillary blot technique (1). The VHb gene probe isolated by miniprep was labeled with biotin using a PolarPlex Kit (Millipore, Bedford, MA). Hybridization and detection reaction were carried out according to the PolarPlex protocol. A permanent image of the hybridization pattern and the VHb gene was obtained by exposing the membrane to an X-ray film.

In a typical autoradiogram obtained as a result of such an experiment, three VHb-CHO clones and the parental CHO-tPA are used. All VHb-CHO clones show two distinct bands hybridized to the VHb probe whereas the parental clone shows one band. The lower band is present in all four clones suggesting that this fragment of DNA exhibits a great deal of homology with the VHb gene and could be the endogenous hemoglobin gene found in Chinese hamster ovary cells. The upper band is present only in VHb-CHO cells indicating that this band corresponds to the VHb gene present in the vector pMSG-VHb that has been integrated in CHO cell chromosome as a result of transfection. Thus, the presence of VHb gene integrated into the chromosome of CHO-VHb cells has been established.

EXAMPLE 14 - OXYGEN-BINDING PROTEIN EXPRESSION IN CHO CLONES

Cell extracts were prepared for each sample as per the protocol described in Khosla et al. (2). Briefly, cells were harvested by trypsinization. The cell suspension was centrifuged at 2500 rpm for 10 minutes at 4°C. The resulting cell pellet was resuspended in 40 ml lysis buffer (100 Mm Tris pH 8.0, 10 mM NaCl and 10 mM EDTA pH 8.0). This cell suspension was subjected to freeze-thaw cycles 3 times in a dry ice-ethanol and 37°C waterbath for 5 minutes each. The resulting suspension was centrifuged at 12000 rpm for 2 minutes at 4°C. The supernatant was transferred to an Eppendorf tube and stored at -20°C until SDS-PAGE was performed.

SDS-PAGE was carried out using standard protocols using the Protein minigel apparatus (Biorad, Richmond, CA). The proteins were transferred to a nitrocellulose membrane and VHb detection was accomplished by Western analysis explained below. The membrane was incubated for 30 minutes with PBS containing non-fat dried milk to prevent non-specific binding. This was followed by a 30 minute incubation with rabbit anti-serum to VHb. The membrane was then subjected to two 10 minute washes with PBS. The membrane was then incubated with horseradish peroxidase conjugated to anti-rabbit antibody for 30 minutes. After two 10 minute washes with PBS, the membrane was then incubated with the horseradish peroxidase substrate 4-chloro-ß-naphthol for 30 minutes which resulted in appearance of bands corresponding to VHb protein. JM101:pRED2 (2) (this *E. coli* strain expresses high levels of VHb) cell extract was used as VHb standard.

EXAMPLE 15 - INDUCTION OF CLONED VHb EXPRESSION IN RECOMBINANT CHO CELLS

Cells expressing VHb were inoculated at 4 x 10^5 cells/dish. Twenty-four hours post inoculation, dexamethasone (Sigma, St. Louis, MO) was added to each dish at final concentrations ranging from 0 to 2 μM. Cell growth was monitored everyday as described earlier. Cell extracts were prepared for Western blot analysis as described in the previous section. In one experiment, the VHb expression was monitored as a function of dexamethasone concentration (0.01, 0.5, 1.0, and 2.0 μM) after 50 hours of induction. In the other experiment, VHb expression was monitored for a single dexamethasone concentration for induction times of 24, 28, 72, and 96 hours induction period. Initial experiments were done with three VHb-CHO clones described in Example 14, namely, 30, 4 and 19. All three express VHb upon induction. Only results for VHb-CHO clone 30 are discussed below.

The results of Western blots from such experiments show that VHb expression is observed in these cells for all concentrations ranging from 0.1 to 2.0 μM. Moreover, VHb expression is observed in samples with induction times of 24 to 96 hours. The uninduced sample (no dexamethasone) also shows a very faint band corresponding to VHb indicating that the MMTV promoter is slightly leaky under conditions of the experiment. This data shows that these CHO cells express VHb upon induction.

EXAMPLE 16 - EFFECT OF CLONED VHb ON tPA PRODUCTION BY RECOMBINANT CHO CELLS

The clones used for this study were the parental CHO-tPA and the VHb-expressing CHO-tPA clone. For the VHb-expressing cells, dexamethasone concentrations of 0, 0.1 and 0.5 μM were used. These cells were

induced on day 1 of the batch culture. All experiments were carried out using 100 x 20 tissue culture dishes. Cells were inoculated on day 0 at a density of 4 x 10^5 cells per dish. On every day of the 5-day batch culture experiment, one dish was removed from the incubator for measurements of cell number, tPA titers and other supernatant metabolite concentrations. Total cell count was monitored using a Coulter Counter. Viability was measured by the trypan blue exclusion method using a hemocytometer. Supernatant was frozen at -20°C for tPA and metabolite analysis. The tPA production was monitored using an ELISA kit (COALIZA, KabiVitrum, Franklin, OH) according to the standard protocol provided by the manufacturer. tPA concentrations in each sample were calculated by using a calibration curve obtained using standard tPA samples provided in the kit.

The total amount of tPA produced each day was calculated by multiplying the concentration of tPA obtained by the volume of supernatant present in each dish. This was done to account for the progressive reduction in volume of the supernatant due to evaporation of water during the course of the batch culture. On each day, cell extracts were prepared and stored as described earlier for analysis of VHb expression during the batch culture. The day 1 sample from VHb-expressing cells corresponds to the uninduced level of VHb expression whereas the CHO-tPA sample serves as negative control for VHb expression since these cells lack the VHb gene. This experiment was carried out two times in order to establish reproducibility and consistency of our experiments. The results of such an experiment are discussed below.

Figures 4 A, B, C, and 5 A, B and C show the total cell number, total tPA produced and total tPA produced/10^6 cells as a function of batch culture time for these two independent experiments. From both these experiments, it is clear that the specific growth rates of VHb-CHO cells is about 20-30% lower than that of the parental CHO-tPA clone. However, this effect is not due to VHb expression since the uninduced VHb-CHO cells show almost the same growth characteristics as the induced VHb-CHO cells. This effect is probably due to some unknown host-vector interaction that manifests itself as a result of integration of transfected DNA sequences into the host cell chromosomes. However, the tPA productivity characteristics are significantly different in the VHb-CHO clone as compared to the parental CHO-tPA clone. The total tPA amounts as well as the amount of tPA produced per cell are about 50-100% higher in VHb-CHO cells compared to these properties in the parental CHO-tPA cell culture.

References:

1. Maniatis, T., Fritsch, E.F. and Sambrook, J. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989.

2. Khosla, C. and Bailey, J.E. Mol. Gen Genet., 214:158-161 1988.

EXAMPLE 17 - EXPRESSION OF A BACTERIAL HEMOGLOBIN IN STREPTOMYCES ENHANCES CELL GROWTH AND OXYGEN UPTAKE RATES UNDER OXYGEN-LIMITED CONDITIONS.

A plasmid was constructed for the expression of a bacterial hemoglobin in Streptomyces. This plasmid, pWLD5, contains the Vitreoscilla hemoglobin gene and its native transcriptional regulatory sequences [Khosla and Bailey (1988) Mol. Gen. Genet., 214:158] cloned into a common Streptomyces plasmid, pIJ699 [Keiser and Melton (1988) Gene, 65:83]. Specifically

the 1.2 kilobase Hind III/SphI Vitreoscilla DNA fragment containing the hemoglobin gene was first inserted into the HindIII/SphI site of the E. coli plasmid pUC19. This construct was then linearized with Hindiii and ligated into HindIII-cut pIJ699. The resulting plasmid, pWLD5, was stably maintained in both E. coli and Streptomyces lividans.

To investigate the effect of hemoglobin on cell growth rate, S. lividans strain TK64 (pro2, str6, obtained from Dr. David Hopwood, John Innes Institute, Norwich, England) was transformed with pWLD5 DNA. A single thiostrepton-resistant colony, designated TK64:pWLD5, was selected for further experiments. Hemoglobin expression in TK464:pWLD5 was confirmed by Western analysis of total cell protein. A crude cell extract was generated by sonication and the proteins separated by SDS-polyacrylamide gel electrophoresis. The proteins were then electrotransferred to nitrocellulose membrane and screened with polyclonal antiserum generated against pure Vitreoscilla hemoglobin. A hemoglobin band of identical molecular weight as pure hemoglobin was detected in the cell extracts. Hemoglobin expression appeared to be constitutive as the levels were similar in cells sampled from any stage of growth. Expression of functional hemoglobin was demonstrated by a carbon monoxide difference spectrum technique [Webster and Liu (1974) J. Biol. Chem. 249:4257].

To investigate the effect of hemoglobin expression on cell growth and respiration, TK64:pWLD5 was compared with the plasmid-free strain (TK64) under two culture conditions corresponding to high and low aeration. The culture medium used for the experiment was as follows: 3% dextrose, 2% N-Z amine Type I, 1% yeast

extract, and 1% v/v trace elements mix (0.1% $FeSO_4 \cdot 7H_2O$, 0.1% $MnSO_4 \cdot 7H_2O$, 0.0025% $CuCl_2 \cdot 2H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, 0.00056% H_3BO_3, 0.002% $ZnSO_4 \cdot 7H_2O$, 0.0019% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$). 5 ug/mL of thiostrepton was added to the TK64:pWLD5 culture. The first condition (high aeration) was a 50 mL culture volume in a 250 mL unbaffled erlenmeyer flask shaken at 250 rpm at 300C. The second condition (low aeration) was a 75 culture volume in a 250 mL unbaffled erlenmeyer flask shaken at 150 rpm at 30°C. With high aeration, the two strains had similar maximum specific growth rates (0.22-0.24 h^{-1}) but the plasmid-free strain reached a higher final cell density (OD_{590}=7.0) compared to TK64:pWLD5 (OD_{590}=5.0). With lower aeration, however, TK64:pWLD5 reached a higher final cell density (OD_{590}=1.95) than the plasmid-free strain (OD_{590}=1.25). This represents a 56% increase in the final cell density in cells expressing hemoglobin under reduced aeration conditions. The maximum specific growth rates of the two strains were similar (0.10-0.11 h_{-1}) under reduced aeration. Hemoglobin expression levels in the two strains were similar throughout the experiment as demonstrated by Western analysis.

Oxygen uptake rates (OUR's) were compared between TK64:pWLD5 and the plasmid-free strain throughout this experiment. Cells were removed at various times, washed, and resuspended in fresh medium at an OD_{590} of 0.10. The OUR's were then measured using a Yellow Springs instruments biological oxygen monitor. The rates were normalized to cell weights and compared throughout the growth curve (Table 1). Although the OUR's of the two strains were similar throughout the experiment with high aeration (Table 1A), they were consistently higher in the hemoglobin-expressing strain with lower aeration, especially at

the later stages of growth (Table 1B). For example, at an OD_{590} of approximately 0.6, the OUR for the plasmid-free strain was 0.22 mM O_2/h-g whereas the OUR for TK64:pWLD5 was 0.29 mM O_2/h-g, a difference of 32%.

This experiment indicates that Streptomyces lividans cells expressing a bacterial hemoglobin grow to significantly higher cell densities and have higher oxygen uptake rates than the non-expressing strain under reduced aeration conditions. A similar plasmid, pWLD15, containing the same Vitreoscilla hemoglobin gene (including its transcriptional regulatory sequence) fragment as that in pWLD5, except that it was cloned into the opposite orientation, also expresses hemoglobin in Streptomyces lividans. This latter finding is evidence that the expression of the hemoglobin gene originates in the inserted fragment (originating from Vitreoscilla) as opposed to elsewhere on the Streptomyces-based pIJ699 plasmid.

Table 1A - High aeration

Strain	O.D.$_{590}$	O.U.R. (mM O_2/h-g)
TK64	0.5	0.32
TK64:pWLD5	0.4	0.35
TK64	0.9	0.32
TK64:pWLD5	0.8	0.33
TK64	5.0	0.11
TK64:pWLD5	4.6	0.12

Table 1B - Low aeration

Strain	$O.D._{590}$	O.U.R. (mM O_2/h-g)
TK64	0.3	0.35
TK64:pWLD5	0.3	0.42
TK64	0.6	0.22
TK64:pWLD5	0.6	0.29
TK64	2.0	<0.10
TK64:pWLD5	2.0	0.27

EXAMPLE 18 - GROWTH ENHANCEMENT OF HEMOGLOBIN-EXPRESSING STREPTOMYCES GROWN UNDER TWO ADDITIONAL CONDITIONS OF REDUCED OXYGEN.

The enhanced growth of hemoglobin-expressing Streptomyces was examined under two additional conditions of low aeration in shake flask cultures. Strains TK64 (no plasmid) and TK65:pWLD5 were cultured in 12.5 and 25 mL culture volumes in 250 mL flasks for 72 hours at 150 rpm at 30°C. The medium used was the same as in Example 17. The final cell densities were measured at OD_{590}. in the 12.5 mL culture, TK64:pWLD5 reached a final OD_{590} of 5.8 while TK64 reached an OD_{590} of only 4.0, a difference of 45%. In the 25 mL culture, TK64:pWLD5 reached a final OD_{590} of 4.5, while TK64 reached an OD_{590} of only 3.3, a difference of 41%. This experiment indicates that hemoglobin expression benefits Streptomyces cell growth under two additional conditions of reduced culture oxygen.

EXAMPLE 19 - EXPRESSION OF BACTERIAL HEMOGLOBIN IN STREPTOMYCES COELICOLOR.

To demonstrate that Vitreoscilla hemoglobin can be expressed in another streptomycete, a plasmid similar to pWLD5 was constructed by inserting BamHI-linearized pRED2 [Khosla and Bailey (1988) Mol. Gen. Genet. 214:158] into BgIII-digested p1J699. The plasmid pRED2 contains the identical hemoglobin sequence as pWLD5 but contains an additional 1.5 kb

of non-essential DNA. The resultant plasmid, pWLD10, was transformed into Streptomyces coelicolor strain M145 (SCP1-, SCP2- obtained from Dr. David Hopwood, John Innes Institute, Norwich, England) and a single thiostrepton-resistant transformant, designated M145:pWLD10, was selected for further experiments.

M145:PWLD10 cells were grown in liquid culture to exponential phase in 50 mL YEME medium (0.3% yeast extract, 0.5% peptone, 0.3% malt extract, 1% glucose, 34% sucrose, 5 mM $MgCl_2 \cdot 6H_2O$) at 250 rpm at 30°C. A cell extract was prepared by sonication and the proteins separated by SDS-PAGE and screened with anti-Vitreoscilla hemoglobin antiserum. Western analysis indicated that a significant level of hemoglobin of identical molecular weight as pure Vitreoscilla hemoglobin was present in cell extracts of M145:pWLD10 but not in the plasmid-free strain. This indicates that Vitreoscilla hemoglobin is stably expressed in another species of Streptomyces.

These data also indicate that the Vitreoscilla hemoglobin promoter element functions in S. coelicolor to express a heterologous protein. Thus, this promoter functions in different strains of Streptomyces.

EXAMPLE 20 - EXPRESSION OF BACTERIAL HEMOGLOBIN IN STREPTOMYCES COELICOLOR RESULTS IN HIGHER FINAL ANTIBIOTIC LEVELS.

Antibiotic production in Streptomyces coelicolor strains M145 and M145:pWLD10 was compared in a shake flask culture experiment. One mL of exponential phase cells were inoculated into 50 mL of YEME medium (5 ug/ml thiostrepton was added to the M145:pWLD10 culture) in 250 mL unbaffled flasks. The cells were grown at 250 rpm at 30°C. Ten days later the cultures were analysed for the production of the

pigmented antibiotic, undecylprodigiosin. The assay was performed by mixing equal volumes of the culture and 0.1 M NaOH followed by a 30" sonication (50 Watt output) on ice. The sonicate was then filtered through a 0.2 uM nitrocellulose membrane. The OD_{468} of the filtrate, which is a measure of undecylprodigiosin, was then determined. While the hemoglobin-expressing strain had an OD_{468} of 1.4, the non-expressing strain had an OD_{468} of only 0.6. This indicated that greater than twice as much antibiotic is produced in a hemoglobin-expressing strain of Streptomyces.

EXAMPLE 21 - EXPRESSION OF BACTERIAL HEMOGLOBIN IN CORYNEBACTERIA

A plasmid was constructed for the expression of a bacterial hemoglobin in Corynebacteria. This plasmid, pBHb3, contains the Vitreoscilla hemoglobin gene (Khosla and Bailey, Mol. Gen. Genet., 214:158, 1988) cloned into a common Corynebacterium plasmid pBK10. Specifically, a 5.5 kilobase plasmid pINT1 (Khosla and Bailey, J. Mol. Biol., 210:79, 1989) which consists of the E. coli plasmid pBR322 with a 1.2 kilobase insert consisting of the Vitreoscilla hemoglobin gene and the 122 base pair tac promoter (P.L. Biochemicals), was digested with Sal 1 and EcoR1. The plasmid ends were then made blunt by filling in with DNA polymerase 1 (Klenow fragment), and the 1.5 kilobase fragment containing the Vitreoscilla hemoglobin gene, tac promoter and flanking pBR322 sequences was isolated. This fragment was ligated to EcoR1 linearized plasmid pBK10 (Paradis, et al., Gene, 61:199, 1987), the ends of which had also been made blunt. The resulting fragment was made circular with T4 DNA ligase. This plasmid, pBHb3, was transformed into E. coli, and was stably maintained by selection with the antibiotic

kanamycin. Corynebacterium glutamicum strain ATTC 39022, a variant of the wild type C. glutamicum strain ATTC 13032, was transformed with pBHb3 DNA out of E. coli. A single kanamycin resistant colony was isolated. This clone was designated 39022:pBHb3-7. The wild-type C. glutamicum strain ATTC 13032 was then transformed with pBHb3 DNA isolated out of clone 39022:pBHb3-7 and a kanamycin resistant colony, designated 13032:pBHb3 15 was selected for further experiments. Hemoglobin expression in 13032:pBHb3-15 was confirmed by Western analysis of total cell protein. A crude cell extract was generated by sonication and the proteins separated by SDS-polyacrylamide gel electrophoresis. The proteins were then electrotransferred to a nitrocellulose membrane and screened with polyclonal antiserum generated against Vitreoscilla hemoglobin purified from E. coli, harboring plasmid pRED2 (Khosla and Bailey, Mol. Gen. Genet., 1988). A band of identical molecular weight as pure hemoglobin was detected in the cell extracts.

EXAMPLE 22 - EXPRESSION OF A BACTERIAL HEMOGLOBIN IN CORYNEFORMS ENHANCES AMINO ACID YIELD AND PRODUCTIVITY IN SHAKE FLASK CULTURES

Lysine production in Corynebacterium glutamicum ATCC 13287 and in similar cells transformed with the plasmid pBHb3 was compared in a shake flask culture experiment. Equal amounts of exponential phase cells were inoculated into 75 mL (250 mL flasks) of the following medium: glucose, 175 g/L; yeast extract, 2 g/L; ammonium sulphate, 55 g/L; magnesium sulfate (heptahydrate), 0.8 g/L; potassium phosphate, 1 g/L; manganese sulfate (tetrahydrate), 0.01 g/L; ferrous sulfate (heptahydrate), 0.01 g/L; biotin, 100 mg/L; thiamine-HCl, 200 mg/L; L-leucine, L-methionine and L-threonine, 0.001 mM each. The pH of the culture

was maintained at neutrality by adding 50 g/L of calcium carbonate to each flask. The cells were grown at 250 rpm shaking speed and 30°C. Samples were taken at different times, and the culture optical density was measured at 600 nm in a spectrophotometer. An OD 600 of 1.0 was determined to correspond to a dry cell weight of 0.35g/Liter. Glucose and lysine concentrations in the sample supernatants were measured by high performance liquid chromatography. Cell extracts were prepared from samples taken from each time point throughout the experiment. Proteins were separated by SDS-PAGE and screened with antiserum against Vitreoscilla hemoglobin. Western analysis confirmed that hemoglobin was being expressed throughout the experiment. This hemoglobin was demonstrated to be functional by a carbon monoxide difference spectrum technique (Webster and Liu, J. Biol. Chem., 1974).

Table 2-1 shows the results obtained 48 hours after inoculation.

Table 2-1: Effect of hemoglobin expression on the production of L-lysine in C. glutamicum ATCC 13287

Strain	13287:pBHb3	13287
glucose (g/L)	159	155
lysine (g/L)	1.60	1.45
OD (600 nm)	5.14	5.67
cell mass (gdw/L)	1.8	2.0

Although the cell yield per glucose consumed is similar in both strains (110 g/kg vs 100 g/kg), the lysine yield per glucose consumed is 43% higher in the hemoglobin containing cells (100 g/kg va 0.07 g/kg). Also, the lysine produced per cell mass is

25% higher in the hemoglobin containing cells (910 g/kg vs 730 g/kg).

Yields:		
g cells/kg glucose	110	100
g lysine/kg glucose	100	70
g lysine/kg cells	910	730
Productivity:		
g lysine/(kg cells.h)	19	15

This experiment indicates that hemoglobin expression in Coryneform increases lysine yield and productivity.

EXAMPLE 37 - GROWTH ENHANCEMENT OF E. COLI IS DUE TO THE OXYGEN BINDING PROPERTIES OF VITREOSCILLA HEMOGLOBIN

In this experiment we compare the growth properties in shake flasks of the recombinant strains expressing Vitreoscilla hemoglobin (LE392:pINT1) and a truncated version (LE392:pBST) of the E. coli hemoglobin-like protein described in example yy with similar plasmid-containing (LE392:pUC18) and plasmid free (LE392) cells. LE392:pBST cells give a negative CO-binding spectrum indicating that the truncated E. coli hemoglobin protein is not biologically active. Experimental medium and conditions were as described in example 36.

Results:
Cell growth as measured by culture optical density after 24th of inoculation is listed in the following table and graphically in FIG. 7:

	LE392	LE392: pUC18	LE392: pINT1	LE392: pBST
Stationary phase				
cell density	5.00	5.32	8.76	5.24
OD (600 nm)				

Conclusion:

Cells containing a biologically active hemoglobin (LE392:pINT) grow to higher cell densities than cell containing a hemoglobin that does not bind oxygen and thus is biologically inactive (LE392:pBST). Cells expressing inactive hemoglobin reach cell densities similar to control cells containing the parent plasmid pUC18 and to no-plasmid control cells.

EXAMPLE 23 - THE PRESENCE OF ACTIVE VITREOSCILLA HEMOGLOBIN IN CORYNEBACTERIUM GLUTAMICUM ENHANCES LYSINE PRODUCTION, YIELD, AND OXYGEN UPTAKE RATE IN BATCH FERMENTATION

In this example, lysine production by C. glutamicum ATCC 13287 cells transformed with the plasmid pBHb3 and plasmid-free cells was studied in batch fermentation experiments.

C. glutamicum ATCC 13287:pBHb3 and C. glutamicum ATCC 13287:no-plasmid cells were grown in 250-mL shake flasks at 30°C and 250 rpm in the following synthetic medium: glucose, 75 g/L; yeast extract, 2 g/L; ammonium sulfate, 55 g/L; magnesium sulfate (heptahydrate), 0.8 g/L; potassium phosphate, 1 g/L; 2manganese sulfate (tetrahydrate), 0.01 g/L; ferrous sulfate (heptahydrate), 0.01 g/L; biotin, 100 mg/L; thiamine-HCl, 200 mg/L; L-leucine, L-methionine and

L-threonine, 200 mg/L each; p 7.0. These cells were used to seed the fermentors.

Fermentations were conducted in 3-L B. Braum MD fermentors in the medium described above, at 30°C and under constant air sparging (0.5 L/min). Initially, the impeller agitation rate was constant. During that time, the dissolved oxygen concentration gradually decreased. When the dissolved oxygen concentration reached 5% of air saturation, the agitation rate was adjusted by the fermentor controller in order to maintain the dissolved oxygen concentration at 5% of air saturation until the end of the fermentations. The culture pH was maintained at pH 7.0 by the fermentor pH controller by periodic additions of 4 N sodium hydroxide.

Samples were taken at different times throughout the fermentations. Glucose and lysine concentrations were determined HPLC. Optical density was measured at 600 nm using a Beckman spectrometer. An OD600 of 1.0 corresponds to a cell mass of 0.35 g dw/L. Analysis of the off-gas oxygen and carbon dioxide content were done using a Perkin-Elmer MGA 1200 mass spectrometer. Oxygen uptake rates (OUR) and respiration coefficients were calculated using the off-gas data. Hemoglobin expression was stable throughout the fermentation as demonstrated by Western electroblotting of the samples taken. The hemoglobin produced by 13287:pBHb3 was biologically active as demonstrated by carbon monoxide binding assay.

Table 3.1 shows the optical density, glucose and lysine concentration as a function of time for 13287:pBHb3 and 13287:np.

Table 3.1

Time (h)		OD600	glucose (g/L)	lysine (g/L)
10	13287:pBHb3	3.5	64	0.1
	13287:np	3.7	74	0.1
20	13287:pBHb3	23	47	4.4
	13287:np	24	47	4.6
35	13287:pBHb3	35	20	15
	13287:np	36	27	12
45	13287:pBHb3	44	5	22
	13287:np	44	11	16
56	13287:pBHb3	42	0.1	27
	13287:np	43	0.2	21

The cell yield per glucose consumed is similar in both strains: 196 g cells/kg glucose for 13287:pBHb3 and 201 g cells/kg glucose for 13287:np. The lysine yield per glucose consumed is 360 g/kg glucose for 13287:pBHb3 and 289 g/kg for 13287:np. The hemoglobin containing cells have a lysine yield 25% higher than the no-plasmid cells. Also, the lysine produced per cell mass is 31% higher in the hemoglobin-producing cells (1,840 g/kg cells for 13287:pBhb3 vs 1,400 g/kg cells for 13287:np). Accordingly, lysine productivity per cell mass is 31% higher in the hemoglobin containing cells (32.9 g/kg cells/h for 13287:pBHb3 and 25.0 g/kg cells/h for 13287:np).

Table 3.2 shows the results from the off-gas analysis during the lysine production period for the 13287:pBHb3 and 13287:np fermentations.

Table 3.2:

Time (h)		OUR (mmol/L/h)	PQ
35	13287:pBHb3	11.2	0.98
	13287:np	7.60	1.05
45	13287:pBHb3	10.2	1.06
	13287:np	6.40	0.92
56	13287:pBHb3	11.1	1.06
	13287:np	6.60	0.94

During the lysine production phase (35, 45 and 58 h), the oxygen uptake rate (OUR) of 13287:pBHb3 averages 10.8 mmol/L/h, a 57% higher than the average OUR of 13287:np during the same time period (6.87 mmol/L/h).

These results indicate that expression of active hemoglobin in Coryneforms increases lysine yield and productivity, and oxygen uptake rate.

EXAMPLE 24 - CONSTRUCTION OF A TRANSFORMATION VECTOR FOR FILAMENTOUS FUNGI

Plasmid pENT 10 is a vector which contains the VHb gene driven by the strong fungal promoter TR-1 isolated from *Trichoderma reesei*. Selection in fungi is by the Sh *ble* gene product, isolated from *Streptoalloteichus hindustanus*, which confers phleomycin resistance to the host. This gene is driven by the fungal promoter GPD isolated from *Aspergillus nidulans*. For bacterial manipulations, selection is tetracycline resistance.

Construction of vector:

A plasmid which confers Tet resistance (Tet r) was constructed first as a base for manipulation in E. coli. Tet r versus Amp r was chosen for an integration plasmid for *Penicillium* since the Beta-lactamase product from the Amp r gene is destructive to the penicillin product and hence would interfere with penicillin production results.

The Tet r vector was constructed by isolating the Tet r gene from a pBR322 based vector and cloning it into pUC 18 (Yanisch-Perron et al., Gene 33:103 (1985)) so as to disrupt the Amp r gene. The tet r gene carried on pVU-2 on an AvaI fragment was inserted via blunt ends into ScaI-AvaII sites of

pUC 18, which inactivtes the amp r gene. The resulting vector was named pTAS 18R.

The cloning of the VHb gene was accomplished by isolating the VHb gene from plasmid p TacHb (Example 5) via a Xba I-Sph I fragment. The TR-1 promoter fragment was isolated from plasmid pUT 737 (purchased from CAYLA, France) on a Nde I-Spe I fragment. These DNA fragments were ligated to vector pTAS 18R cut with Nde I-Sph I. The resulting intermediate vector was named pENT 1B. The Sh ble gene, GPD promoter and Trp C terminator region were isolated from vector pUT 720 (CAYLA, France) by a Eco RI-Xba I digest, Klenowed, and inserted into the Bam HI site of pENT 1B which had also been treated with Klenow to remove protruding DNA ends and create a blunt end cloning site. The resulting vector was named pENT 10.

EXAMPLE 25 - TRANSFORMATION OF PENICILLIUM CHRYSOGENUM

Transformation into fungi is accomplished by breaking down cell walls via enzymatic digestion to form protoplasts which are then selectively permeable to uptake of exogenous DNA. DNA which is transformed into fungi is not maintained as plasmid DNA as in *E. coli* but integrated into the host genome.

Buffers used in procedure

KMC (osmotic stabilization buffer)
700mM KC1
50mMC CaC12
10 mM MES (buffering agent)
-pH is brought to 5.8 by addition of HCl

Lytic Buffer
50mM Phosphate buffer pH 5.8
700mM KC1

PMC (Aids in uptake of DNA)
50% PEG 8000
50 mM CaC12
10 mM MES
-pH is brought to 5.8 by addition of HCl

NaCl (osmotically stable wash)
0.9% soln

Basic Procedure for transformation:

- inoculate conidia in minimal media (200 ml), supplemented with 2.5 mM CaCl2 and 200µl of a standard trace element solution.

- grow at 28°C for 40 hours.

- mycelia are recovered by filtration through a 30µm filter and washed with a 0.9% solution.

- Protoplasting and transformation procedures were carried out essentially as those described in Cantoral J.M. et al, Bio/Technology, Vol 5, May 1987 pg.494-496.

- Selection of protoplasts was on minimal osmotic media supplemented with 0.5% yeast extract containing 20µg/ml of phleomycin (CAYLA, France) at 28°C and overlayed with the same media in a soft agar base after 24 hours.

EXAMPLE 27 - EXPRESSION OF A BACTERIAL HEMOGLOBIN IN *PENICILLIUM CHRYSOGENUM* IMPROVES FINAL ANTIBIOTIC LEVELS.

In this example, penicillin production was studied in hemoglobin-expressing *P.chrysogenum* and in non-expressing control cells in batch fermentations. The plasmid pENT10 described in Example 24 was used for integration of the *Vitreoscilla* hemoglobin gene into the genome of *Penicillium chrysogenum* ATCC 48271.

Medium and conditions: The seed medium was as follows (per liter): 30 g glucose, 10 g lactose monohydrate, 30 ml corn steep liquor, 2 g ammonium sulfate, 5 g calcium carbonate, 0.5 g potassium dihydrogen phosphate, 10 g Pharmamedia, 10 g yeast extract. Two hundred ml of the seed medium was inoculated with spores to a final concentration of 1 x 10^8 spores/ml. The seed cultures were grown at 30°C for 48 hours at 220 rpm and were used to inoculate 2-liter fermentors. The fermentation medium consisted of the following (per liter): 120 g lactose monohydrate, 27.5 g Pharmamedia, 10 g ammonium sulfate, 10 g calcium carbonate, 10 g lard oil, 0.5 g fermentation cultures were grown at 30°C for 24 hours in B. Braun MD fermentors. The air flow rate was maintained at 1 l/min and the dissolved oxygen was controlled at 30% of air saturation throughout the fermentation. At 24 hours, the temperature was reduced to 25°C, keeping all other conditions the same, and a phenylacetic acid (potassium salt) feed was initiated at a rate of 0.07 g/h. Throughout the duration of the fermentation, the off-gas was analyzed by mass spectrometry (model 1200, Perkin-Elmer, USA).

High pressure liquid chromatography (HPLC) was employed to determine the species and concentrations of penicillin present in the fermentation broths.

Samples for HPLC analysis were removed from the fermentors, filtered through sterile 0.2 um filters and frozen at -20°C or assayed immediately. The filtrate penicillin concentrations were measured using a Shimadzu HPLC system. Penicillin G obtained from Sigma was used as the standard. Samples, after dilution with buffer A, were analyzed for penicillin using an AXXI-CHROM C-18 (ODS) column (Cole Scientific, Calabasas, CA). Buffer A was a 0.015 M solution of ammonium acetate in 5% (v/v) methanol. Dilutions of the samples and the standard preparations were made with this buffer. Buffer B was a 0.015 M solution of ammonium acetate in 30% (v/v) methanol. The peaks were eluted over a binary gradient and were detected at 220 nm in a UV spectrophotometer (model SPD-6AV, Shimadzu, Japan). Peak areas were calculated by an integrator (model CR501, Shimadzu, Japan). The levels of penicillin concentrations obtained in shake flask cultures were elevated by greater than 20% in strains transformed with the VHb gene relative to control strains.

EXAMPLE 28 - CLONING OF HMP PROTEIN IN E. coli AND TRUNCATED hmp GENES

A plasmid was constructed for the purpose of enhancing the expression of the hmp gene (hemoprotein) in E. coli. The hmp gene (S.G. Vasudevan, et al. Mol Gen Genet, 1991, vol, 226: 49-58) encodes a 44 kDa protein which has dihydropteridine reductase (DHPR). The hmp amino acid sequence bears great similarity to that of the Vitreoscilla hemoglobin (VHb) gene having 46% identity through the first 146 amino acids.

Two synthetic oligomers were prepared based upon the dmp nucleotide sequence as published by S.G. Vasudevan. A 25-oligomer encodes the first 18

nucleotides of the hmp gene starting from the first ATG, preceded by a synthetic restriction enzyme site (Xbal) and consists of the sequence 5' CTC-TAG-AAT-GCT-TGA-CGC-TCA-AAC-C 3'. The second 25-obigomer encodes the complement to the end of the hmp gene starting with the first stop codon, preceded by a synthetic restriction enzyme site (Kpn 1) and consists of the sequence 5' AGG-TAC-CTT-ACA-GCA-CCT-TAT-GCG-A 3'. These two oligomers were used to perform a PCR reaction in which the template was genomic DNA isolated from E. coli strain K-12. A 1200bp PCR product was generated which restriction analysis showed to be the hmp gene. This PCR product was digested with Xba 1 and Kpn 1, and ligated to the plasmid pTacVhb from which the VHb sequence has been removed by digestion with Xba 1 and Kpn 1. The ligation mix was used to transform competent E. coli ceells and transformants were selected by resistance to the antibiotic amplicilin. Several resistant clones were geneerated from which plasmid DNA was isolated. Restriction analysis showed one of the plasmids to be correct and it waS designatted pTac-HMP. The expression of pTac-HMP in E. coli was confirmed by a carbon monoxide difference spectrum technique (Webster and Liu, J. Biol. Chem., 1974). The difference spectrum of control cells not transformed showed no Co-binding activity, while cells transformed with pTac-HMP showed the difference spectrum characteristic of cells expressing functional hemoglobin and hemoglobin-like (hmp) proteins.

Two additional plasmids were constructed which are identical to pTac-HMP through that portion of the hmp sequence which is similar to the VHb sequence, but which have the dis-similar hmp sequences removed. Plasmid pTac-Bst was constructed by digesting

pTac-HMP with Xba 1 and Mlu 1. An approx. 400bp fragment was isolated which consists of the first portion of the hmp gene from the first ATG to a unique Mlu 1 site, and encodes the first 118 amino acids of the hmp protein. This fragment was ligated into plasmid pTac-VHb which had been cut with Xba 1 and Mlu1 in-order to remove the first 120 amino acids of the VHb gene. Plasmid pTac-Bst thus contains a DNA sequence which encodes the first 118 amino acids of the hmp gene followed by the last 26 amino acids of the VHb gene. These two segments are joined at a unique Mlu 1 site which both genes have in-common. Plasmid pTac-Bst was transformed into E. coli cells and expression was confirmed by Western analysis. A crude cell extract was generated by sonication and the proteins separated by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to a nitrocellulose membrane and screened with polyclonal antiserum generated against Vitreoscilla hemoglobin. A band of identical molecular weight as pure hemoglobin was detected in the cell extract. A carbon monoxide difference spectrum was performed, however no functional protein could be detected by this method.

The third plasmid was constructed by using PCR to generate a truncated version of the hmp gene and then ligating the product into pTacVHb from which the VHb sequences had been removed. Specifically, a 23-oligomer was designed which encodes the complement to 17 nucleotides of the hmp DNA sequence from a region aprox. 7 amino acids past the end of the portion of the hmp gene which is similar to the VHb gene. These 17 nucleotides are preceded by an additional 6 nucleotides which encode the restriction enzyme site Bsu36I. The Bs36 I site was designed to encode a stop codon. This 23-oligomer consists of the

sequence 5' ACC-TTA-GGC-TTT-GCT-GGC-GTT-TT 3'. A PCR reaction was carried out using this 23-oligomer and the 25-oligomer which codes for the first portion of the hmp gene, and using plasmid pTac-HMP DNA for template. A PCR product of aprox. 440bp was generated which restriction analysis showed to be a portion of the hmp gene. The 3' end of this product was digested with the restriction enzyme Bsu36 I and made blunt by filling in with DNA polymerase I (Klenow fragment). This DNA fragment was then cut with the restriction enzyme Xba 1 and ligated into plasmid pTacVHb which had been cut with EcoR1, been made blunt-ended by filling-in with Klenow, and then digested with Xba 1 to remove the VHb sequence. The ligation mix was transformed into competent E. coli cells and a transformant which contained a correct plasmid construct as judged by restriction analysis was isolated. This plasmid was designated pTac-K12Hb. The E. coli clone transformed with pTac-K12Hb was used for two experiments. Western analysis was performed to confirm the expression of the truncated hmp gene, however no hybridization to Vitreoscilla hemoglobin anti-sera could be detected. A carbon monoxide difference spectrum was performed, but no functional hmp protein could be detected.

EXAMPLE 29 - CONSTRUCTION OF TRUNCATED VERSIONS OF E. COLI hmp AND VECTORS FOR EXPRESSION IN E. COLI

Two plasmids were constructed which are identical to pTac-HMP through that portion of the hmp sequence which is similar to the VHb sequence, but which have the dis-similar hmp sequences removed. Plasmid pTac-Bst was constructed by digesting pTac-HMP with Xba 1 and Mlu 1. An aprox. 400bp fragment was isolated which consists of the first portion of the hmp gene from the first ATG to a unique Mlu 1 site, and

encodes the first 118 amino acids of the hmp protein. This fragment was ligated into plasmid pTacVHb which had been cut with Xba 1 and Mlu 1 in order to remove the first 120 amino acids of the VHb gene. Plasmid pTac-Bst thus contains a DNA sequence which encodes the first 118 amino acids of the hmp gene followed by the last 26 amino acids of the VHb gene. These two segments are joined at a unique Mlu 1 site which both genes have in-common. Plasmid pTac-Bst was transformed into E. coli cells and expression was confirmed by Western analysis. A crude cell extract was generated by sonication and the proteins separated by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to a nitrocellulose membrane and screened with polyclonal antiserum generated against Vitreoscilla hemoglobin. A band of identical molecular weight as pure hemoglobin was detected in the cell extract. A carbon monoxide difference spectrum was performed, however no functional protein could be detected by this method.

The third plasmid was constructed by using PCR to generate a truncated version of the hmp gene and then ligating the product into pTacVHb from which the VHb sequences had been removed. Specifically, a 23-oligomer was designed which encodes the complement to 17 nucleotides of the hmp DNA sequence from a region aprox. 7 amino acids past the end of the portion of the hmp gene which is similar to the VHb gene. The 17 nucleotides are preceded by an additional 6 nucleotides which encode the restriction enzyme site Bsu36I. The Bsu36 I site was designed to encode a stop codon. This 23-oligomer consists of the sequence 5' ACC-TTA-GGC-TTT-GCT-GGC-GTT-TT 3'. A PCR reaction was carried out using this 23-oligomer and the 25-oligomer which codes for the first portion of the hmp gene, and using plasmid pTac-HMP DNA for

template. A PCR product of aprox. 440bp was generated which restriction analysis showed to be a portion of the hmp gene. The 3' end of this product was digested with the restriction enzyme Bsu36 I and made blunt by filling in with DNA polymerase I (Klenow fragment). This DNA fragment was then cut with the restriction enzyme Xba 1 and ligated into plasmid pTacVHb which had been cut with EcoR1, been made blunt-ended by filling-in with Klenow, and then digested with Xba 1 to remove the VHb sequence. The ligation mix was transformed into competent E. coli cells and a transformant which contained a correct plasmid construct as judged by restriction analysis was isolated. This plasmid was designated pTac-K12Hb. The E. coli clone transformed with pTac-K12Hb was used for two experiments. A carbon monoxide difference spectrum showed no detectable functional activity for the truncated hmp protein.

EXAMPLE 29 CONSTRUCTION OF VECTOR FOR EXPRESSION OF HORSE-HEART MYOGLOBIN IN C. glutamicum

A 7.3Kb plasmid was constructed for the expression of horse Myoglobin in Coryneform bacteria. Plasmid pGYM was digested with restriction enzymes Xba1 and SSP1. The larger of two fragments was gel isolated. This fragment contained all of the myoglobin gene and some pUC sequence but no promoter. This fragment was ligated to a fragment of plasmid pTac-Bst which had been first digested with Mlu1, made blunt with DNA polymerase 1 (Klenow fragment), then digested with Xba1, the smaller of two fragments having been gel isolated. This smaller fragment consists of the Tac promoter and some pUC sequence. The two fragments were ligated together and transformed into competent E. coli cells. Transformants were screened for plasmid DNA consisting of the pUC vector having a

Tac-Myoglobin insert. One such transformant was found and its plasmid DNA, pUC-TacMyo, was isolated for further manipulations. Plasmid pUC-TacMyo was digested with restriction enzyme Hind III. This liberated an 800bp fragment consisting of the myoglobin gene preceded by the Tac promoter. This fragment was gel isolated, made blunt using Klenow, and ligated with a deleted version of plasmid pFS1 (pFS1 del). Plasmid pFS1 del had been prepared by first digesting Nhe1 to remove the Tac-VHb sequence, then filling in the ends with Klenow. The pFS1 del - TacMyo. ligation mix was transformed into competent Coryneform strain ATTC 13287. One Coryneform transformant showed a reddish pigment and plasmid isolated from this transformant was determined to be a correct construction by restriction analysis and in particular by the presence of a unique EcoR1 site which is present in the myoglobin sequence but is not present in the VHb sequence. This Coryneform transformant was designated 13287:pFS-TacMyo. The expression of functional myoglobin in 13287:pFS-TacMyo was demonstrated by a carbon monoxide difference spectrum.

EXAMPLE 30 - CONSTRUCTION OF VECTOR FOR THE EXPRESSION OF SOYBEAN LEGHEMOGLOBIN IS *E. coli*

A 5.2Kb plasmid was constructed for the expression of a soybean leghemoglobin protein (LHbc1) in *E. coli*. This plasmid, pKK-LHbc1, contains the cDNA to soybean leghemoglobin c1 cloned into the expression vector pKK 233-2 (Pharmacia). Specifically, a 3.5Kb plasmid pCD1 which consists of the *E. coli* plasmid pBluescript SK (Stratagene) with the 600bp insert consisting of a cDNA sequence to the soybean leghemoglobin c1 gene, was used as template in a polymerase chain reaction (PCR). In order to perform

the PCR two DNA oligomers were designed which were complementary to the 5' and the 3' ends of the leghemoglobin cDNA sequence. Each oligomer also included an additional 6 bases which code for a unique restriction site. The oligomer which is complementary to the 5' end of the + strand of leghemoglobin c1 cDNA starts with a Nco1 restriction site and consists of the sequence 5'-tcc-atg-ggt-gct-ttc-act-gat-aa-3'. The oligomer which is complementary to the 3' end of the - strand of leghemoglobin c1 cDNA starts with a Hind III site and consists of the sequence 5'-caa-gct-ttt-ttt-ttt-ttt-ttt-ttt-t-3'. A polymerase chain reaction amplified the 600bp LHb c1 sequence resulting in a PCR product having a Nco1 site at the 5' end and a Hind III site at the 3' end. This PCR product was ligated with the cloning plasmid pCR 1000 (Invitrogen). This ligation mix was transformed into competent E. coli cells and transformants were selected by resistance to the antibiotic kanamycin. Plasmid DNA was isolated from several transformants and screened for the presence of full-length LHb c1 cDNA sequence by restriction analysis and gel electrophoresis. One clone was found to be correct and was designated plasmid pTA-LHbc1. Plasmid pTA-LHbc1 was linearized with Spe1 which cuts 40bp past the 3' terminus of the LHbc1 insert. The plasmid ends were then made blunt by filling in with DNA polymerase 1 (Klenow fragment). The LHbc1 sequence was removed from the pCR 1000 vector by digestion with Nco1, and this 600bp fragment was purified by agarose gel electrophoresis. An expression vector, pKK 233-2 (Pharmacia) was prepared by first linearizing with Hind III and filling in the ends with DNA polymerase 1 (Klenow fragment), then digesting with Nco1. The purified 600bp LHbc1 fragment and the linearized pKK 233-2 were ligated together and transformed into competent

E. coli cells. Transformants were selected by resistance to the antibiotic ampicillin. Plasmid DNA was isolated from several transformants and screened for the presence of full-length LHbc1 sequence by restriction analysis and gel electrophoresis. One clone was found to be correct and designated clone pKK-LHbc1. Plasmid pKK-LHbc1 was transformed into E. coli strains JM101 and LE 392. A single ampicillin resistant transformant of each strain was isolated and designated JM101: pKK-LHbc1, and LE 392: pKK-LHbc1. Expression of functional leghemoglobin was confirmed in both transformants by carbon monoxide binding assay (Webster, D.A., 1974, J. Biol. Chem. 249:4257-4260). A crude cell extract was prepared from each transformant as well as control cells by sonication. Cell debris was removed by centrifugation and each extract was divided into two aliquots. One aliquot from each sample was left exposed to air while the second aliquot was saturated with carbon monoxide. After thirty minutes all air-exposed and carbon monoxide-saturated aliquots were reduced by the addition of dithionite. The absorbance spectra from 400 to 500 nm was measured for each aliquot. For each sample the difference in aliquots was plotted. Cell extracts of JM101: pKK-LHbc1 and LE 392: pKK-LHbc1 showed a difference in absorbance between the air-exposed and carbon monoxide-saturated aliquots at aprox. 417nm which indicates the presence of functional leghemoglobin protein.

EXAMPLE 31 - EXPRESSION OF THE OXYGEN BINDING PROTEINS MYOGLOBIN, LEGHEMOGLOBIN, AND HAEMOPROTEIN ENHANCES CELL DENSITY IN E. COLI SHAKE FLASK CULTURES

Cell densities achieved by E. coli strain LE 392 and LE 392 transformed with plasmids pINT1, pGYM (see example D), pTac-HMP, and pKK-LHbc1 was compared in a shake flask culture experiment. Equal amounts of exponential phase cells were inoculated into 12.5 ml (250ml flasks) of the following medium: 10g/L yeast extract, 0.5g/L KH2PO4, 1.5g/L K2HPO4, 4g/l (NH4)2SO4, 5g/L glucose, 1.0ml/L 1M MgSO4 7H2O, 0.0.5 ml 100mM MgC12 2H2O, and 0.2ml 100mM FeC13. The medium was supplemented with 1.0ml/L of the following trace metal solution: in 100mls 2.7g FeC13 6H2O, 0.2g ZnCl, 0.2g CaC12 2H2O, 0.2g NaMoO4 (VI), 0.19g CuSO4 5H2O, and 0.05 g boric acid. The medium was further supplemented with 1.0ml of the following vitamin solution: in 500mls 0.21g riboflavin, 2.7g pantothenic acid, 3.0g niacin/nicotanic acid, 0.7g pyridoxine, 0.03g biotin, and 0.02g folic acid. The pH of the medium was adjusted to pH7.3 and maintained with 12.1g/L Trisma base. The cells were grown at 350rpm shaking speed and 37 deg. C. Cell density was monitored by measuring absorbance at 600nm in a spectrophotometer (OD600). Cell density was monitored at hourly intervals from 19 to 23 hours after inocultion. Maximum cell density was determined to be when OD600 did not increase within one hour. The maximum cell density obtained for the parent E. coli strain LE 392 and four transformants is shown in Table C-1.

Table C-1: Effect of the expression of four oxygen-binding proteins on maximum cell density attained by E. coli strain LE 392.

Strain	Protein Product	Final OD600	% of LE 392
LE 392		9.2	100.0
LE 392: pINT1	Vitreoscilla hemoglobin	12.2	132.6
LE 392: pGYM	Horse heart myoglobin	12.3	133.7
LE 392: pTacHMP	E. coli haemoprotein	9.8	106.5
LE 392:pKK-LHbc1	Soybean leghemoglobin	10.7	116.3

After termination of the shake flask experiment cell extracts were prepared from each of the five strains and assayed for expression of functional oxygen-binding activity. Oxygen-binding activity in the four transformants was confirmed by the carbon monoxide difference spectrum technique of Webster (1974, J. Biol. Chem. 249:4257-4260). As can be seen, all the globin-containing strains outgrew the control strain. These proteins are diverse in nature, being from animal, plant, and microbial origin. Because the common function of all the proteins is to reversibly bind oxygen, it is probable that oxygen-binding is the primary property of these proteins that results in growth improvement.

EXAMPLE 32 - CONSTRUCTION OF PLASMID FOR EXPRESSION OF MYOGLOBIN

A plasmid used for the expression of myoglobin in Escherichia coli was constructed in the following manner. The structural gene encoding horse heart myoglobin was synthesized based on its amino acid sequence [Eur. J. Biochem. 11, 267-277, 1969]. The purified gene was then inserted into the E.coli pEMBL18+ [Methods Enzymol. 155, 111-119, 1987]. The myoglobin gene is under the control of the tac promoter. The resulting plasmid, called pGYM, was

transformed into E. coli strain LE392 (supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1).

The presence of functional myoglobin in LE392:pGYM cells was confirmed by two methods. First, LE392:pGYM cells were significantly darker than LE392 cells without plasmid or containing a plasmid without the myoglobin gene. This dark color is due to the high intracellular concentrations of heme which is bound to the myoglobin protein. Second, carbon monoxide difference spectral analysis indicated the presence of an abundant intracellular CO-binding protein in LE392:pGYM cells but not in the control cells. Thus, functional myoglobin has been demonstated to be expressed in E coli cells using the recombinant plasmid pGYM.

EXAMPLE 33 - EXPRESSION OF MYOGLOBIN IN *E.COLI*

The aim of this study was to show that expression of the oxygen-binding protein myoglobin in E. coli provides a growth benefit to cells as has been documented for Vitreoscilla hemoglobin (VHb) [Nature 331, 633-635, 1988, Patent # 5,049,493]. The following strains were tested in shake-flask cultures:

Strain	Comment
LE392	Host strain (without plasmid)
LE392:pUC18	Plasmid control strain
LE392:pGYM	Myoglobin-expressing strain
LE392:pINT1	Hemoglobin-expressing strain [J. Mol. Biol. 210, 79-89, 1989]

The experimental cultures contained 12.5 mL of glucose semi-defined medium (see EXAMPLE 32) in 250 mL erlenmeyer flasks shaken at 250 rpm in a New Brunswick G24 incubator at 37°C. The flasks were seeded with a 0.5% (v/v) inoculum of late expoinential-phase cells. The culture optical

densities (ODs) were measured between 22-24 hrs, when the cultures had reached their maximum ODs. The maximum cell ODs are shown below:

Strain	Comment
LE392	6.06
LE392:pUC18	6.02
LE392:pGYM	9.70
LE392:pINT1	10.14

As can be seen, the myoglobin (LE392:pGYM) and the hemoglobin LE392:pINT1) cells outgrew the control cells by 60% and 67%, respectively. This result indicates that myoglobin provides a similar benefit to cell growth as Vitreoscilla hemoglobin. Because the function of both proteins is to reversibly bind oxygen, it is probable that oxygen binding is the primary proeprty of these proteins that reuslsts in growth improvement.

EXAMPLE 34 - EXPRESSION OF MYOGLOBIN IS NOT STRAIN SPECIFIC

To test whether expression of myoglobin results in improved growth of an E. coli strain with a different genetic background than LE392, pGYM was transformed into strain MG1655 (K12, lambda-). A growth experiement similar to the previous example was performed. The only difference in the growth conditions ws that Tris-HC1 was not added to the medium. This resulted in slightly lower maximum ODs. The pGYM (myoglobin) strain was compared to the pUC18 control strain. The maximum culture ODs are shown below.

Strain	Maximum OD (A600)
MG1655:pUC18	5.23
MG1655:pGYM	6.63

Thus, E. coli strain MG1655 expressing myoglobin demonstrates a 27% improvement in growth over the control, indicating that the beneficial effect of myoglobin is not strain specific.

EXAMPLE 36 - GROWTH OF ENHANCEMENT OF *E. COLI* IN BATCH FERMENTATION

The growth properties are compared of the recombinant strains expressing Vitreoscilla hemoglobin (LE392:pINT1), horse heart myoglobin (LE392:pGYM), soybean leg hemoglobin (LE392:pKK-LHbc1), and E. coli hemoprotein (LE392:pTac-HMP) with similr plasmid-containing (LE392:pUC18) and plasmid free (LE392) cells under typical batch fermentation conditions.

Cells were grown in B. Braun MD fermentors at 37±0.5°C and pH of 7±0.05 with a working volume of 2 L in the semi-defined medium described in Example C. A constant air flow rate of 2 L/min and agitator speed of 700 rpm were maintained throughout each run. In all cases, the dissolved oxygen (DO) levels remained above 5% of air saturation under those aeration conditions.

Results:
The growth parameters measured for the six strains are listed in the following table:

	LE392: pUC18	LE392: pINT1	LE392: pGYM	LE392: pKK-LHbc1	LE392: pTac-HMP	LE392 (PLASMID FREE)
Log-phase growth rate (1/h)	0.76	0.95	0.95	1.03	0.87	0.92
Stationary phase cell density OD (600 nm)	4.2	7.6	7.5	7.6	7.4	7.5
Final total protein (g/L)	0.96	1.15	1.22	1.30	1.36	1.28

Conclusion:

Cells containing hemoglobins grow faster and to higher cell densities than comparable plasmid-containing controls.

EXAMPLE 36 - THE PRESENCE OF OXYGEN BINDING HEMOGLOBINS IN CORYNEBACTERIUM GLUTAMICUM ENHANCES L-LYSINE PRODUCTION AND YIELD.

In this example, production of lysine in batch termination by C. glutamicum ATCC 13287 transformed with plasmid pFS1 (Vitreoscilla hemoglobin) and with plasmid pMYO (myoglobin) was measured and compared to that of the parent strain (no plasmid).

C. glutamicum ATCC 13287:pFS1, C. glutamicum ATCC 13287:pMYO, and C. glutamicum ATCC 13287:no-plasmid cells were grown in 250-mL shake flasks at 30°C and 250 rpm in the following synthetic medium: glucose, 60 g/L; yeast extract, 2 g/L; ammonium sulfate, 55 g/L; magnesium sulfate (heptahydrate), 0.8 g/L; potassium phosphate, 1 g/L; manganese sulfate (tetrahydrate), 0.01 g/L; ferrous sulfate (heptahydrate), 0.01 g/L; biotin, 100 μg/L; thiamine-HCl, 200 μg/L; L-leucine, L-methionine and L-threonine, 200 mg/L each; pH 7.0. These cells were used to seed the fermentors.

Fermentations were conducted in 3-L B. Braun MD fermentors in the medium described above, at 30°C and udner constant air sparging (1.0 L/min). Initially, the impeller agitation rate was constant. During that time, the dissolved oxygen concentration gradually decreased. When the dissolved oxygen concentration reached 5% of air saturation, the agitation rate was adjusted by the fermentor controller in order to maintain the dissolved oxygen concentration at 5% of air saturation until the end of the fermentations. The culture pH was maintained

at pH 7.0 by the ferrrmentor pH controller by periodic additions of 4 N sodium hydroxide.

Samples were taken at different times throughout the fermentations. Glucose and lysine concentrations were determined by HPLC. Optical density was measured at 600 nm using a Beckman spectrometer. An OD_{600} of 1.0 corresponds to a cell mass of 0.35 g dw/L. Vitreoscilla hemoglobin and myoglobin expression was stable throughout the fermentation as demonstrated by Western electroblotting of the samples taken. The oxygen-binding proteins produced by both 13287.pFS1 and the myoglobin produced by 13287:pMYO were biologically active as demonstrated by carbon monoxide binding assay.

Table 2.1 shows the optical density, glucose and lysine concentration as a function of time for 13287:pFS1, 13287:pMYO, and 13287:np.

Table 2.1:

Time (h)		OD_{600}	glucose (g/L)	lysine (g/L)
7.5	13287:pFS1	16.3	43.8	0.8
	13287:pMYO	17.0	37.2	1.0
	13287:np	23.4	38.5	1.5
24	13287:pFS1	45.2	7.7	8.2
	13287:pMYO	48.4	8.4	8.1
	13287:np	46.0	7.1	6.6
28	13287:pFS1	52.4	0.0	12.0
	13287:pMYO	51.6	0.0	11.3
	13287:np	53.2	0.0	9.3

The cell yield per glucose consumed is similar in all the strains: 305 gcells/kgglucose for 13287:pFS1, 302 gcells/kgglucose for 13287:pMYO, and 310 gcells/kgglucose for 13287:np. The lysine yield per glucose consumed is 200 g/kkglucose for 13287:pFS1, 188 g/kgglucose for 13287:pMYO, and 155 g/kgglucose

for 13287:np. Vitreoscilla hemoglobin-containing cells have a lysine yield 29% higher than the no-plasmid cells, and myoglobin-containing cells have a lysine yield 21% higher than the no-plasmid cells. Lysine productivity per cell mass is 31% higher in the Vitreoscilla hemoglobin containing cells (23.4 g/kgcells/h for 13287:pFS1 and 17.9 g/kgcells/h for 13287:np). Also, lysine productivity per cell mass is 25% higher in the myoglobin containing cells (22.3 g/kgcells/h for 13287:pMYO and 17.9 g/kgcells/h for 13287:np).

These results indicate that expression of an active oxygen binding protein such as Vitresocilla hemoglobin or myoglobin in Coryneforms increases lysine accumulation; yield, and productivity.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
Bailey, J. et al.

(ii) TITLE OF INVENTION:
ENHANCEMENT OF CELL GROWTH BY EXPRESSION AS A CLONED OXYGEN-BINDING PROTEINS

(iii) NUMBER OF SEQUENCES:
2

(iv) CORRESPONDENCE ADDRESS:

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(C) CITY:
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(D) STATE:
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(E) COUNTRY:
United States of America

(F) ZIP:

94104-2878

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE:

Diskette, 3.50 inch, 1.2 Kb storage

(B) COMPUTER:

IBM PC/XT/AT compatable

(C) OPERATING SYSTEM:

MS Dos 3.3

(D) SOFTWARE:

Word Perfect 5.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

07/887,169

07/741,789

07/342,451

07/151,256

07/113,014

(B) FILING DATE:

05/20/92

08/6/91

10/21/88

02/28/88

10/23/87

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184-996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:

745 base pairs

(B) TYPE:

nucleic acid

(C) STRANDEDNESS:

single

(D) TOPOLOGY:

linear

(ii) MOLECULE TYPE:
cDNA to genomic RNA
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(v) ORIGINAL SOURCE:

(A) ORGANISM:
(B) STRAIN: Vitreoscilla sp.
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENT STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:
(vi) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE: pRED2
(ix) PUBLICATION INFORMATION:
(A) AUTHORS: Wakabayashi et. al.
(B) TITLE:
(C) JOURNAL: Nature
(D) VOLUME: 322
(E) ISSUE:
(F) PAGES 483
(G) DATE: 1986
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES:

(x) A SEQUENCE DESCRIPTION:SEQ ID NO:1:

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ATGAACTTAA  GGAAGACCCT  C ATG TTA GAC CAG CAA ACC ATT AAC ATC ATC AAA GCC ACT  180
                          Met Leu Asp Gln Gln Thr Ile Asn Ile Ile Lys Ala Thr

GTT CCT GTA TTG AAG GAG CAT GGC GTT ACC ATT ACC ACG ACT TTT TAT AAA AAC TTG TTT  240
Val Pro Val Leu Lys Glu His Gly Val Thr Ile Thr Thr Thr Phe Tyr Lys Asn Leu Phe

GCC AAA CAC CCT GAA GTA CGT CCT TTG TTT GAT ATG GGT CGC CAA GAA TCT TTG GAG CAG  300
Ala Lys His Pro Glu Val Arg Pro Leu Phe Asp Met Gly Arg Gln Glu Ser Leu Glu Gln


CCT AAG GCT TTG GCG ATG ACG GTA TTG GCG GCA GCG CAA AAC ATT GAA AAT TTG CCA GCT  360
Pro Lys Ala Leu Ala Met Thr Val Leu Ala Ala Ala Gln Asn Ile Glu Asn Leu Pro Ala

ATT TTG CCT GCG GTC AAA AAA ATT GCA GTC AAA CAT TGT CAA GCA GGC GTG GCA GCA GCG  420
Ile Leu Pro Ala Val Lys Lys Ile Ala Val Lys His Cys Gln Ala Gly Val Ala Ala Ala

CAT TAT CCG ATT GTC GGT CAA GAA TTG TTG GGT GCG ATT AAA GAA GTA TTG GGC GAT GCC  480
His Tyr Pro Ile Val Gly Gln Glu Leu Leu Gly Ala Ile Lys Glu Val Leu Gly Asp Ala

GCA ACC GAT GAC ATT TTG GAC GCG TGG GGC AAG GCT TAT GGC GTG ATT GCA GAT GTG TTT  540
Ala Thr Asp Asp Ile Leu Asp Ala Trp Gly Lys Ala Tyr Gly Val Ile Ala Asp Val Phe

ATA CAA GTG GAA GCA GAT TTG TAC GCT CAA GCG GTT AGA T AAAGTTTCAG  GCCGCTTTCA  600
Ile Gln Val Glu Ala Asp Leu Tyr Ala Gln Ala Val Glu

GGACATAAAA  AACGCACCAT  AAGGTGGTCT  TTTTACGTCT  GATATTTACA  CAGCAGCAGT          660

TTGGCTGTTG  GCCAAAACTT  GGGACAAATA  TTCGCCTGTG  TAAGAGCCCG  CCGTTGCTGC          720

GACGTCTTCA  GGTGTGCCTT  GGCAT                                                  745
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(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:
146 amino acids

(B) TYPE:
amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY:
linear

(ii) MOLECULAR TITLE:
protein

(A) DESCRIPTION:
Vitreoscilla hemoglobin

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: INTERNAL FRAGMENT

(iv) ORIGINAL SOURCE:

(A) ORGANISM: Vitreoscilla sp.

(ix) FEATURE:

(A) NAME/KEY: Vitreoscilla hemoglobin protein

(B) LOCATION:

(C) IDENTIFICATION METHOD: Western analysis

(D) OTHER INFORMATION: binds oxygen

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Wakabayashi et. al.

(B) TITLE:

(C) JOURNAL: Nature

(D) VOLUME: 322

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(F) PAGES 483

(G) DATE: 1986

(xi) A SEQUENCE DESCRIPTION:SEQ ID NO:2:

```
                    5                   10
    Met-Leu-Asp-Gln-Gln-Thr-Ile-Asn-Ile-Ile-
                    15                  20
 5  Lys-Ala-Thr-Val-Pro-Val-Leu-Lys-Glu-His-
                    25                  30
    Gly-Val-Thr-Ile-Thr-Thr-Thr-Phe-Tyr-Lys-
                    35                  40
    Asn-Leu-Phe-Ala-Lys-His-Pro-Glu-Val-Arg-
10                  45                  50
    Pro-Leu-Phe-Asp-Met-Gly-Arg-Gln-Glu-Ser-
                    55                  60
    Leu-Glu-Gln-Pro-Lys-Ala-Leu-Ala-Met-Thr-
                    65                  70
15  Val-Leu-Ala-Ala-Ala-Gln-Asn-Ile-Glu-Asn-
                    75                  80
    Leu-Pro-Ala-Ile-Leu-Pro-Ala-Val-Lys-Lys-
                    85                  90
    Ile-Ala-Val-Lys-His-Cys-Gln-Ala-Gly-Val-
20                  95                  100
    Ala-Ala-Ala-His-Tyr-Pro-Ile-Val-Gly-Gln-
                    105                 110
    Glu-Leu-Leu-Gly-Ala-Ile-Lys-Glu-Val-Leu
                    115                 120
25  Gly-Asp-Ala-Ala-Thr-Asp-Asp-Ile-Leu-Asp-
                    125                 130
    Ala-Trp-Gly-Lys-Ala-Tyr-Gly-Val-Ile-Ala-
                    135                 140
    Asp-Val-Phe-Ile-Gln-Val-Glu-Ala-Asp-Leu-
30                  145                 150
    Tyr-Ala-Gln-Ala-Val-Glu
```

CLAIMS

WHAT IS CLAIMED IS:

1. A recombinant-DNA method for production of an oxygen-binding protein in a host cell grown in the presence of of oxygen, said host cell being chosen and derived from a selection of cells consisting of microorganisms and cells of multi-cellular organisms selected from the group consisting of animals, plants and insects comprising:

(a) preparing a portable DNA sequence capable of directing a host cell to produce a protein having oxygen-binding activity;

(b) introducing said portable DNA sequence into a host cell capable of expressing said protein;

(c) growing the host cell under conditions appropriate for expression of said protein.

2. A method according to Claim 1 wherein said host cell is grown in culture.

3. A method according to Claim 1 further comprising the steps:

(d) in either order:

(i) harvesting said protein; and

(ii) permitting said protein to assume an active structure whereby it possesses oxygen-binding activity.

4. The method of Claim 1, wherein said portable DNA sequence is introduced directly and integrated into the chromosome of a host cultured cell.

5. The method of Claim 1, wherein said portable DNA sequence is introduced into said host cultured cell by the following steps:

(a) cloning the portable DNA sequence into a vector capable of being transferred into and replicating in a host cell, such vector containing operational elements for the portable DNA sequence;

(b) transferring the vector containing the portable DNA sequence and operational elements into a host cultured cell capable of expressing said oxygen-binding protein;

(c) culturing the host cell under conditions appropriate for replication and propagation of the vector and expression of said protein.

6. The method of Claim 1 wherein said host microorganisms are selected from the group consisting of bacteria, fungi, molds, and yeast.

7. The method of Claim 6 wherein said host organism comprises yeast.

8. The method of Claim 1 wherein said protein is harvested prior to being permitted to assume said active structure.

9. The method of Claim 1, wherein said protein is allowed to assume said active structure prior to being harvested.

10. The method of Claim 2, wherein said vector is amplified in a microbial host prior to transfer into the host cell.

11. A method according to Claim 5 wherein said host comprises Penicillium chrysogenum.

12. A process for subjecting the expression of a selected DNA sequence encoding an oxygen-binding protein to external control under given environmental conditions which comprises the steps of:

(a) providing at least one selected isolated structural gene that is responsive to a Vitreoscilla hemoglobin promoter/regulator DNA sequence under the given environmental conditions; and

(b) operatively fusing the selected structural gene with said promototer/regulator DNA sequence.

13. The process of Claim 12, wherein said structural gene is transcriptionally responsive.

14. The process of Claim 12, wherein said structural gene is translationally responsive.

15. A method for expressing an oxygen-binding protein in a host E. coli cultured cell, comprising:

(a) introducing into said host cultured cell a DNA expression vector containing a DNA sequence according to SEQUENCE No. 1, and DNA sequences coding for said oxygen-binding protein; and

(b) growing said host cultured cell in an appropriate medium and environment and isolating said oxygen-binding protein.

16. The method of Claim 15, wherein said DNA expression vector is a portable DNA sequence, and is introduced directly and integrated into the chromosome of a host cultured cell.

17. A method for increasing the growth characteristics, including the growth yield, the growth rate, and the achievable cell density under controlled circumstances, of a host cultured cell grown in the presence of oxygen, said host cultured cell being chosen and derived from a selection of cultured cells consisting of microorganisms and cells obtained from multi-cellular organisms selected from the group consisting of animals, plants and insects comprising:

(a) preparing a portable DNA sequence capable of directing said host cultured cell to produce a protein having at least some oxygen-binding activity;

(b) introducing said portable DNA sequence into a host cultured cell capable of expressing at least some oxygen-binding protein;

(c) culturing the host cell under conditions appropriate for expression of the protein.

18. The method of Claim 17, wherein said portable DNA sequence is introduced directly and integrated into the chromosome of a host cultured cell.

19. The method of Claim 17, wherein said portable DNA sequence is introduced into said host cultured cell by the following steps:

(a) cloning the portable DNA sequence into a vector capable of being transferred into and replicating in a host cell, such vector containing operational elements for the portable DNA sequence;

(b) transferring the vector containing the portable DNA sequence and operational elements into a host cultured cell capable of expressing at least some of the oxygen-binding protein; and

(c) culturing the host cell under conditions appropriate for replication and propagation of the vector and expression of the protein.

20. A method for increasing the production of proteins, both those normally made and those expressed as a result of genetic engineering; biopolymers; amino acids; antibiotics; and other metabolic products, of a host cultured cell grown in the presence of oxygen, said host cultured cell being chosen and derived from a selection of cultured cells consisting of microorganisms and cells obtained from multi-cellular organisms selected from the group consisting of animals, plants and insects comprising:

(a) preparing a portable DNA sequence capable of directing a host cultured cell to produce a protein having oxygen-binding activity;

(b) introducing said portable DNA sequence into a host cultured cell capable of expressing at least some oxygen-binding protein;

(c) culturing the host cell under conditions appropriate for expression of the oxygen-binding protein.

21. A method according to Claim 20 wherein production of said oxygen-binding protein increases production of L-lysine in said host cell.

22. A method according to Claim wherein productions of said oxygen-binding protein increases production of Penicillin G in said host cell.

23. A method for transporting and supplying oxygen to oxygen-requiring processes and operations, comprising:

(a) preparing a portable DNA sequence capable of directing a host cultured cell to produce a protein having oxygen-binding activity;

(b) introducing said portable DNA sequence into a host cultured cell capable of expressing at least some oxygen-binding protein;

(c) culturing the host cell under conditions appropriate for expression of the protein;

(d) effectively delivering said host cell or a preparation from said host cell containing the oxygen-binding protein to said oxygen-requiring process.

24. A method for the binding and removal of oxygen from an environment comprising:

(a) preparing a portable DNA sequence capable of directing a host cultured cell to produce a protein having oxygen-binding activity;

(b) introducing said portable DNA sequence into a host cultured cell capable of expressing at least some oxygen-binding protein;

(c) culturing the host cell under conditions appropriate for expression of the oxygen-binding protein; and

(d) effectively delivering said host cell or a preparation from said host cell containing the oxygen-binding protein to said oxygen-containing environment.

25. A method for increasing the growth characteristics, including the growth yield, the growth rate, and the achievable cell density under controlled circumstances, of a cell in culture, said host cultured cell being chosen and derivatized from a selection of cultured cells consisting of microorganisms and cells obtained from multicellular organisms selected from the group consisting of animals, plants, and insects, said cell being capable of producing an endogenous protein having at least some oxygen-binding activity, comprising culturing said host cell under conditions appropriate for expression of said oxygen-binding protein.

26. A method for increasing the production of proteins, both those normally made and those expressed as a result of genetic engineering,

biopolymers; amino acids; antibiotics; and other metabolic products, of a host cultured cell grown in the presence of oxygen, said host cultured cell being chosen and derived from a selection of cultured cells consisting of microorganisms and cells obtained from multi-cellular organisms selected from the group consisting of animals, plants and insects, said cell being capable of producing an endogenous a protein having at least some oxygen-binding activity, comprising culturing said host cell under conditions appropriate for expression of said oxygen-binding protein.

27. A method for the binding and removal of oxygen from an environment comprising the introduction of a host cultured cell to said oxygen-containing environment, said host cultured cell expressing a protein which possesses at least some oxygen-binding activity.

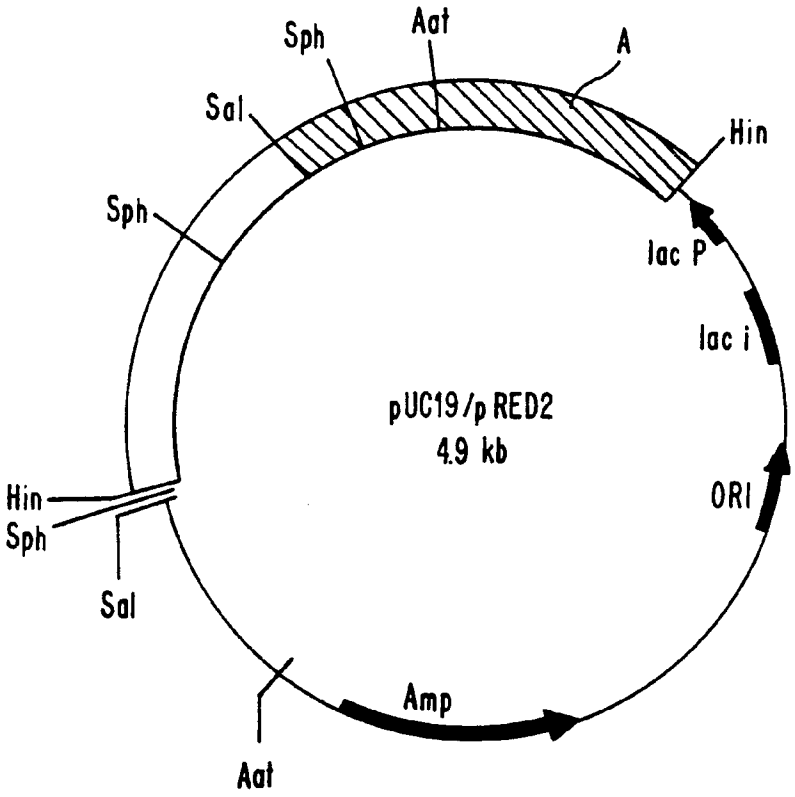

Sph
Aat
A
Sal
Hin
Sph
lac P
lac i
pUC19/p RED2
4.9 kb
ORI
Hin
Sph
Sal
Amp
Aat

FIG. 1.

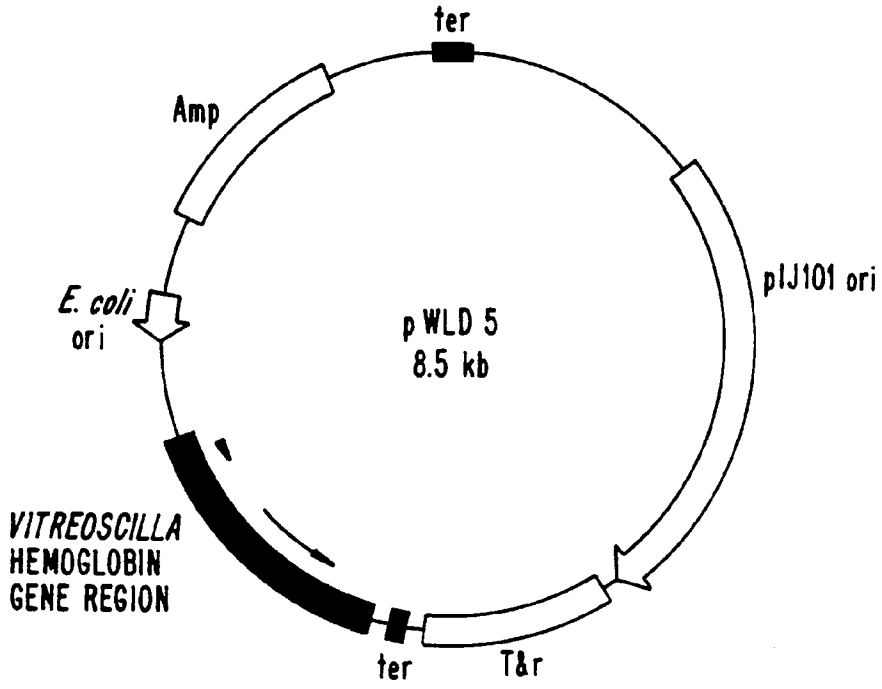

ter
Amp
pIJ101 ori
E. coli
ori
p WLD 5
8.5 kb
VITREOSCILLA
HEMOGLOBIN
GENE REGION
ter
T&r

FIG. 2A.

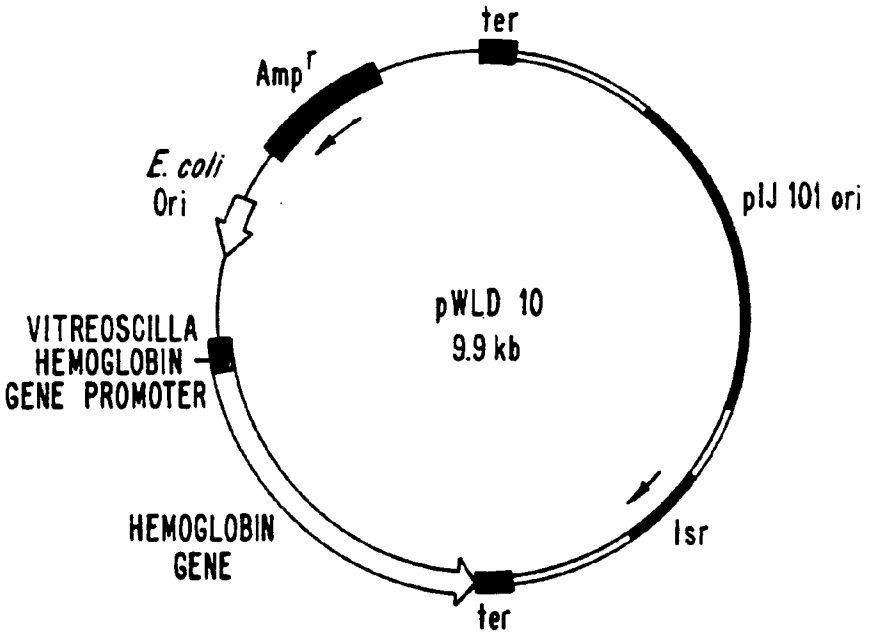

ter
Amp r
E. coli
Ori
pIJ 101 ori
pWLD 10
9.9 kb
VITREOSCILLA
HEMOGLOBIN
GENE PROMOTER
HEMOGLOBIN
GENE
Isr
ter

FIG. 2B.

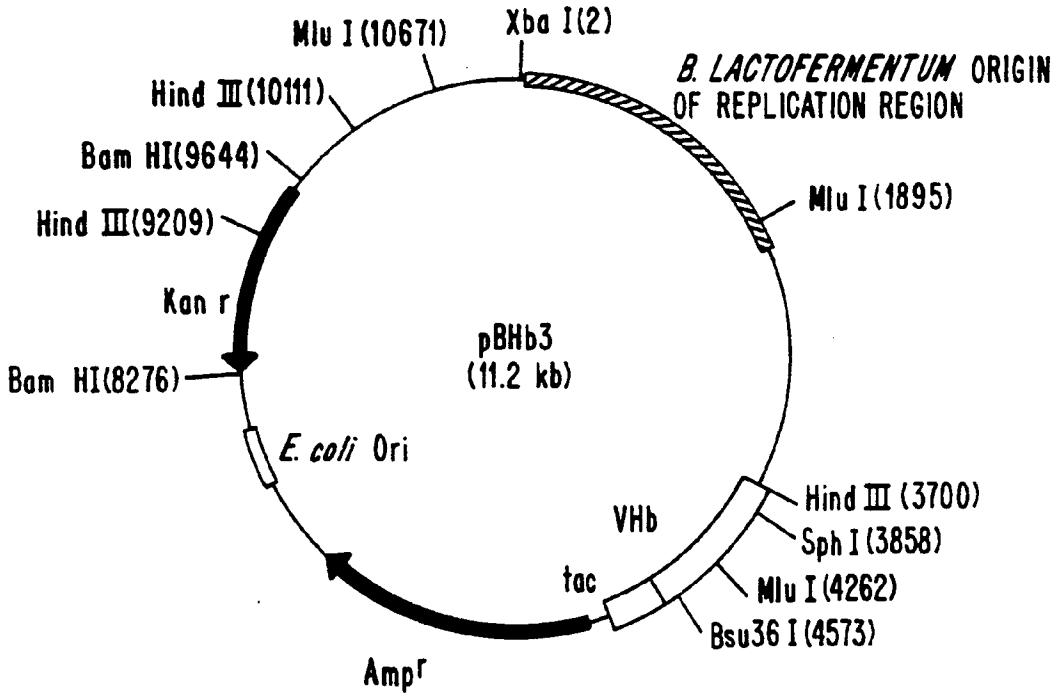

Mlu I (10671)
Xba I(2)
B. LACTOFERMENTUM ORIGIN
OF REPLICATION REGION
Hind III(10111)
Bam HI(9644)
Mlu I(1895)
Hind III(9209)
Kan r
pBHb3
(11.2 kb)
Bam HI(8276)
E. coli Ori
Hind III (3700)
VHb
Sph I (3858)
tac
Mlu I (4262)
Bsu36 I (4573)
Amp r

FIG. 3.

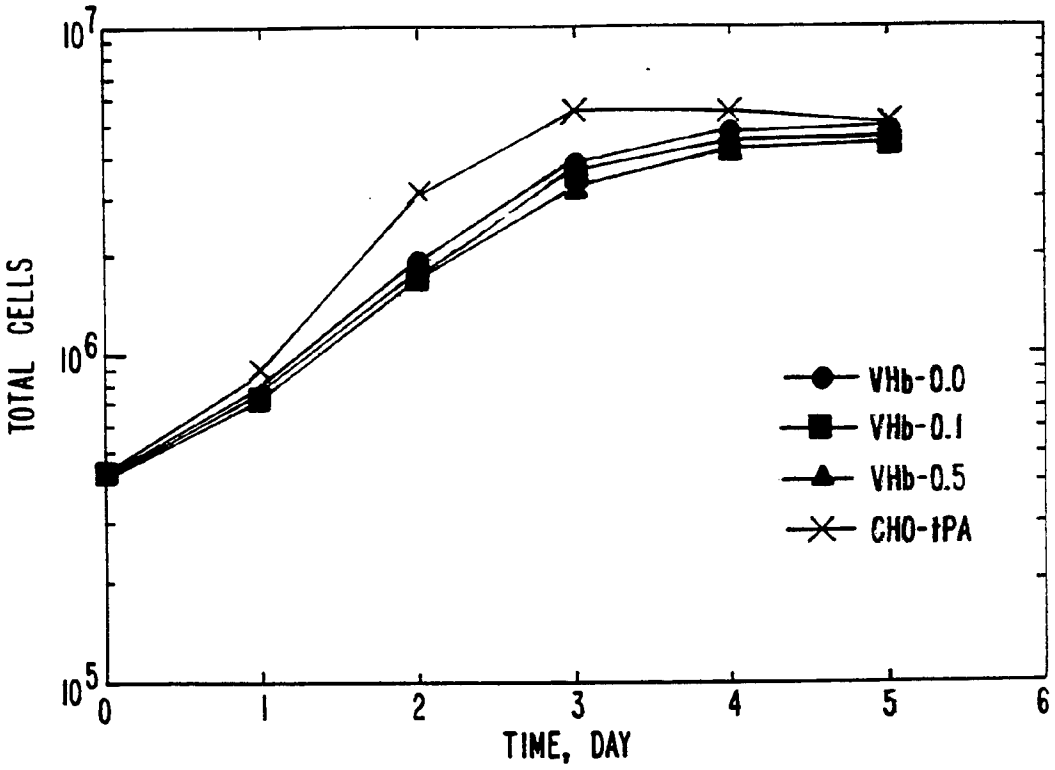

TOTAL CELLS
10^7
10^6
10^5
0
1
2
3
4
5
6
TIME, DAY
VHb-0.0
VHb-0.1
VHb-0.5
CHO-tPA

FIG. 4A.

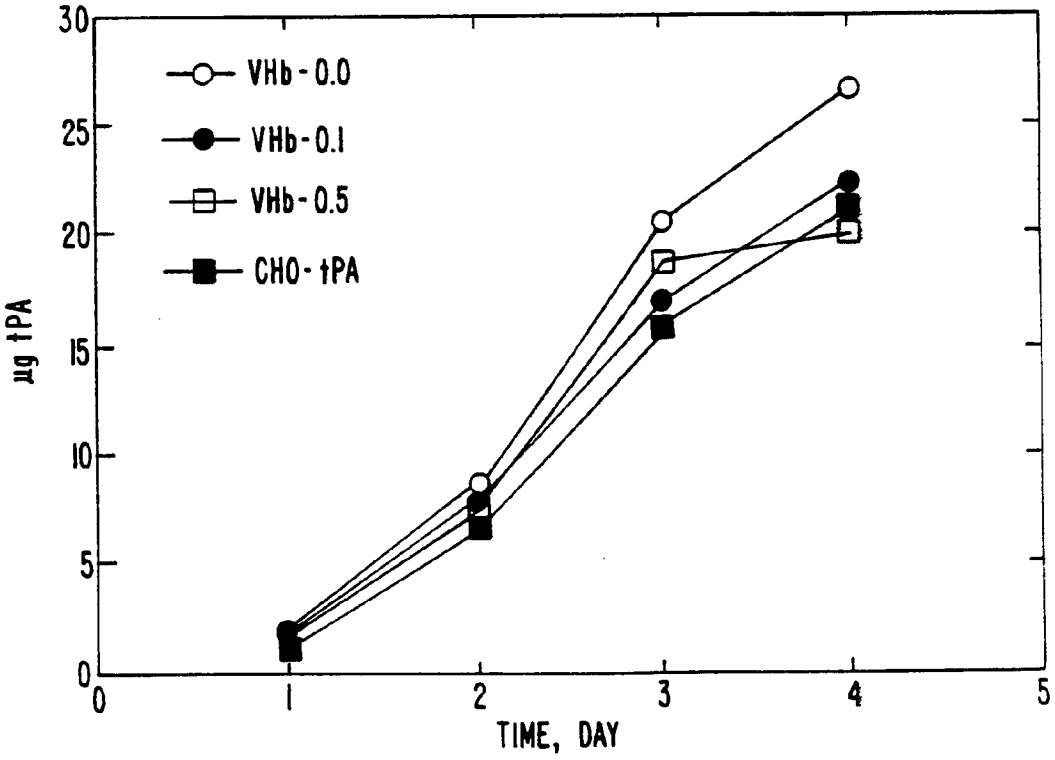

µg tPA
30
25
20
15
10
5
0
0
1
2
3
4
5
TIME, DAY
VHb-0.0
VHb-0.1
VHb-0.5
CHO-tPA

FIG. 4B.

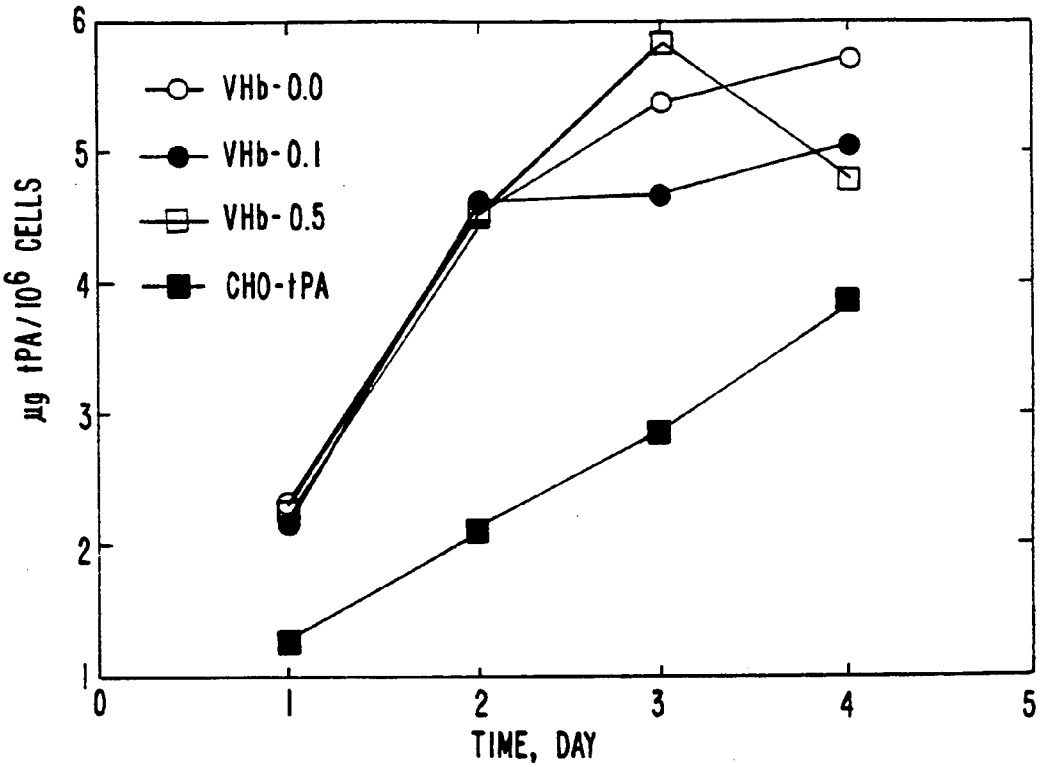

VHb-0.0
VHb-0.1
VHb-0.5
CHO-tPA
µg tPA/10^6 CELLS
TIME, DAY
0
1
2
3
4
5
1
2
3
4
5
6

FIG. 4C.

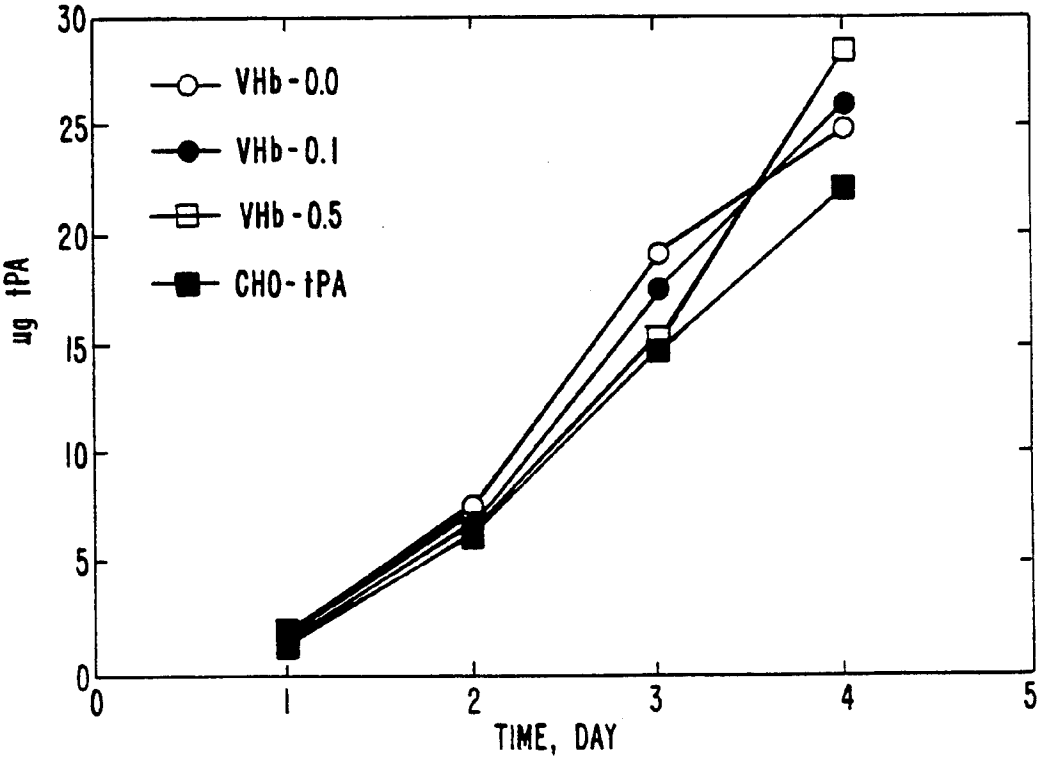

VHb-0.0
VHb-0.1
VHb-0.5
CHO-tPA
µg tPA
TIME, DAY
0
5
10
15
20
25
30
0
1
2
3
4
5

FIG. 5A.

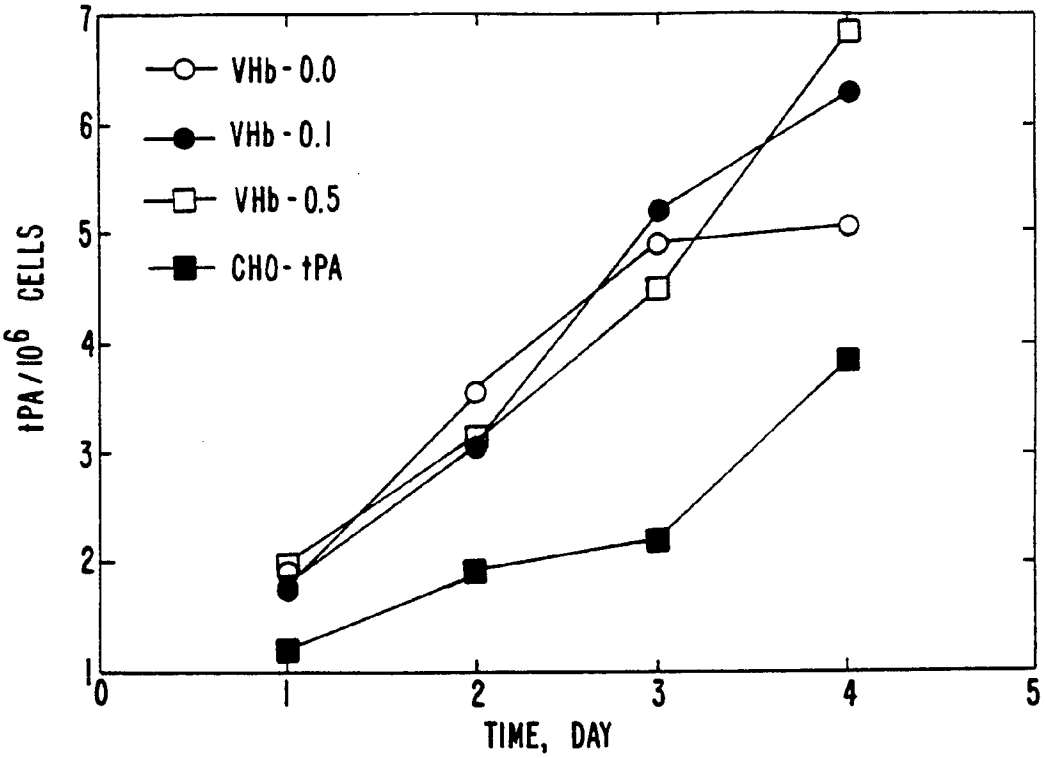

VHb - 0.0
VHb - 0.1
VHb - 0.5
CHO - tPA
tPA / 10^6 CELLS
TIME, DAY
0
1
2
3
4
5
6
7

FIG. 5B.

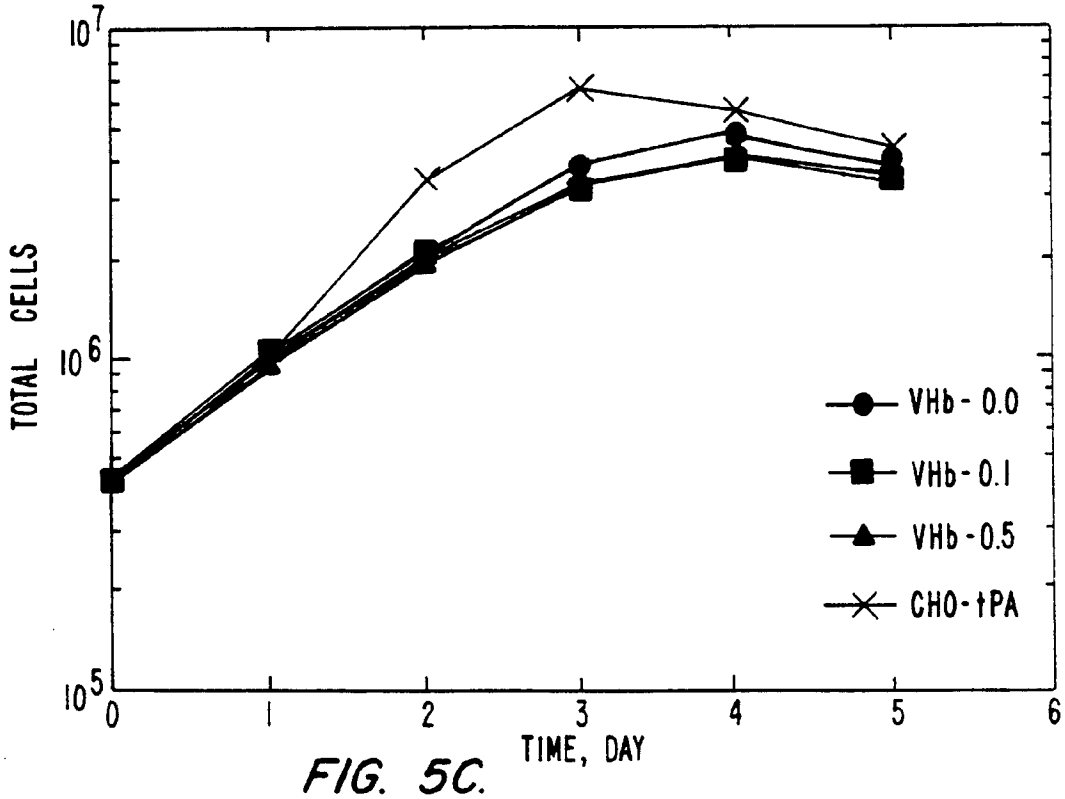

VHb - 0.0
VHb - 0.1
VHb - 0.5
CHO - tPA
TOTAL CELLS
TIME, DAY
10^5
10^6
10^7
0
1
2
3
4
5
6

FIG. 5C.

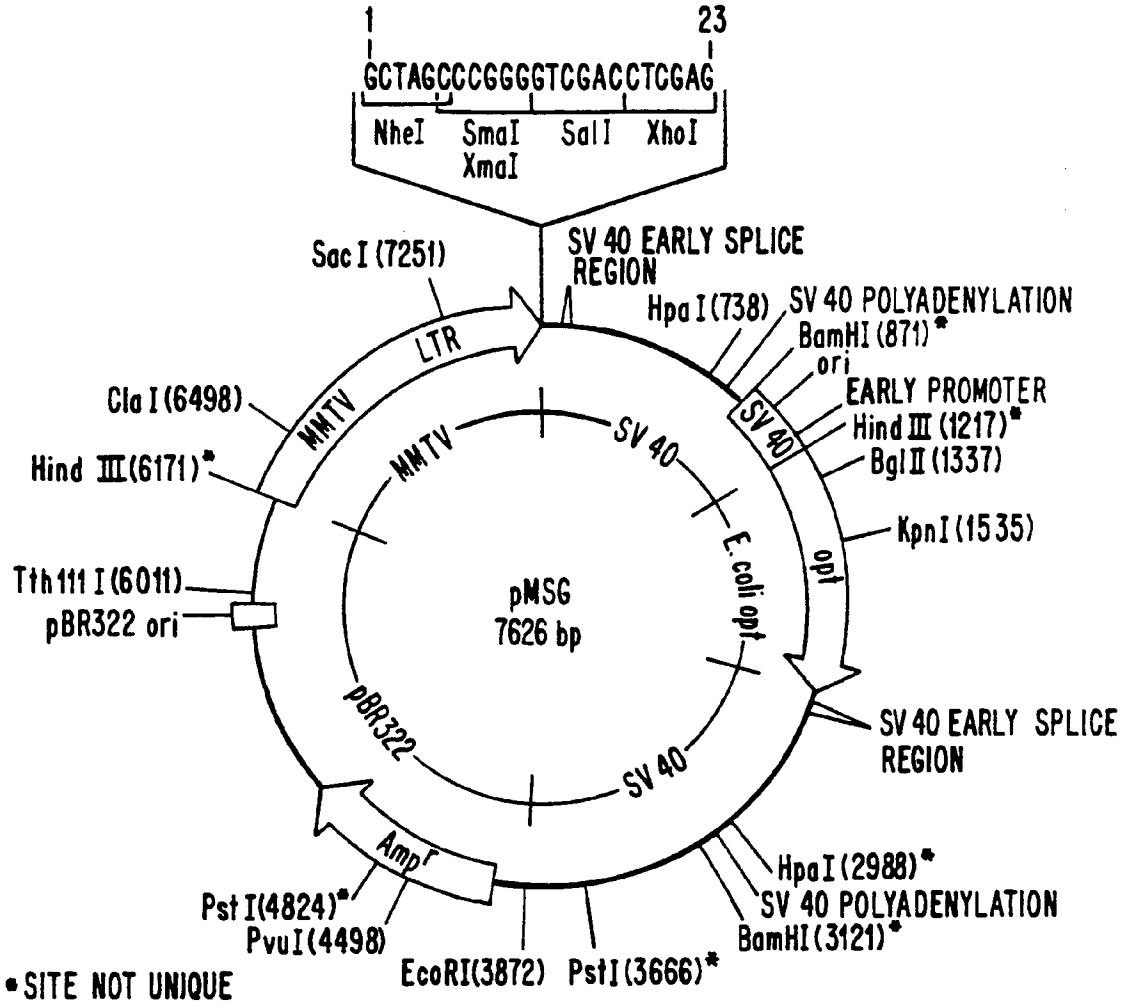

1
23
GCTAGCCCGGGGTCGACCTCGAG
NheI
SmaI
XmaI
SalI
XhoI
SV 40 EARLY SPLICE REGION
Sac I (7251)
Hpa I (738)
SV 40 POLYADENYLATION
BamHI (871)*
ori
EARLY PROMOTER
Hind III (1217)*
Bgl II (1337)
Kpn I (1535)
Cla I (6498)
Hind III (6171)*
Tth 111 I (6011)
pBR322 ori
LTR
MMTV
MMTV
SV 40
E. coli opt
opt
pMSG
7626 bp
pBR322
SV 40
Amp^r
SV 40 EARLY SPLICE REGION
Hpa I (2988)*
SV 40 POLYADENYLATION
BamHI (3121)*
Pst I (4824)*
Pvu I (4498)
EcoRI (3872)
Pst I (3666)*
* SITE NOT UNIQUE

FIG. 6.

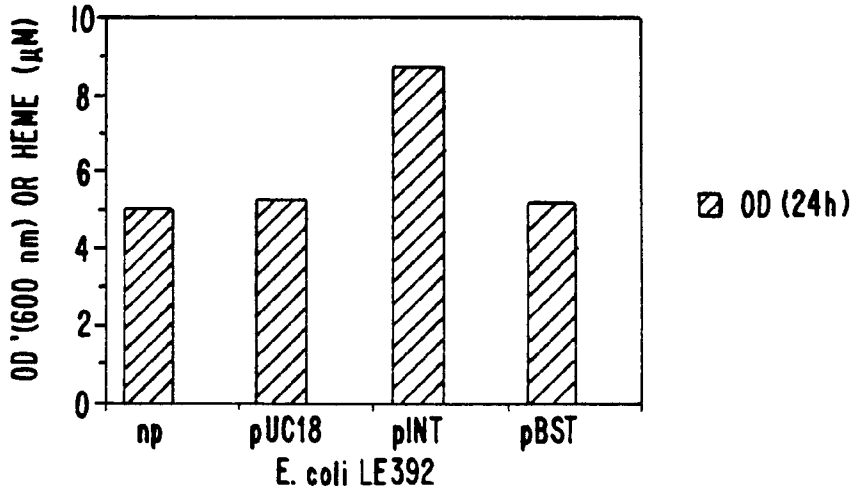

OD '(600 nm) OR HEME (μM)
10
8
6
4
2
0
np
pUC18
pINT
pBST
E. coli LE392
OD (24h)

FIG. 7.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05527

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12P 21/00, 1/00, 13/04, 13/08, 37/00; C12N 1/00, 15/00
US CL :435/69.1, 172.3, 41, 43, 106, 115, 317.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 172.3, 41, 43, 106, 115, 317.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

U.S. Automated Patent System, Biosis, Derwent Biotechnology Abstracts. Keywords: Vitreoscilla, hemoglobin, haemoglobin, oxygen, penicillin, chrysogenum, recombinant, vector, expression.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A, 89/03883 (KHOSLA ET AL) 05 MAY 1989, see claims 122, 37-39, 50-58.	1-10, 12-20, 23-27
X	WO, A, 92/03546 (SANDER ET AL) 05 MARCH 1992, see claims 8-12, 14-17	1, 2, 6, 20, 21, 26
X Y	WO, A, 91/06628 (HUGHES ET AL) 16 MAY 1991, see claims 6-8, 21-23, 27-29	1,2,6,17,20,25,26 11, 22
X Y	Bio/Technology, vol. 9, issued May 1991, Magnolo et al, "Actinorhodin production by Streptomyces coelicolor and growth of Streptomyces lividans are improved by the expression of a bacterial hemoglobin", pages 473-476, see entire document.	1,2,5,6,10,17,19, 20,25,26 4, 11, 18, 22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search
15 JULY 1993

Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05527

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biotechnology and Bioengineering, vol. 22, no. 2, issued February 1980, Ryu et al, "Quantitative Physiology of Penicillium chrysogenum in Penicillin Fermentation", pages 289-298, see page 293, lines 1-12.	11, 22
Y	Biotechnology and Bioengineering, vol. 36, issued 20 December 1990, Ho et al, "Enhancing Penicillin Fermentations by Increased Oxygen Solubility Through the Addition of n-Hexadecane", pages 1110-1118, see abstract.	11, 22
Y	EP, A, 0,260,762 (MARTIN ET AL) 23 MARCH 1988, see entire document.	4, 11, 18, 22

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

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(71) Applicant: E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

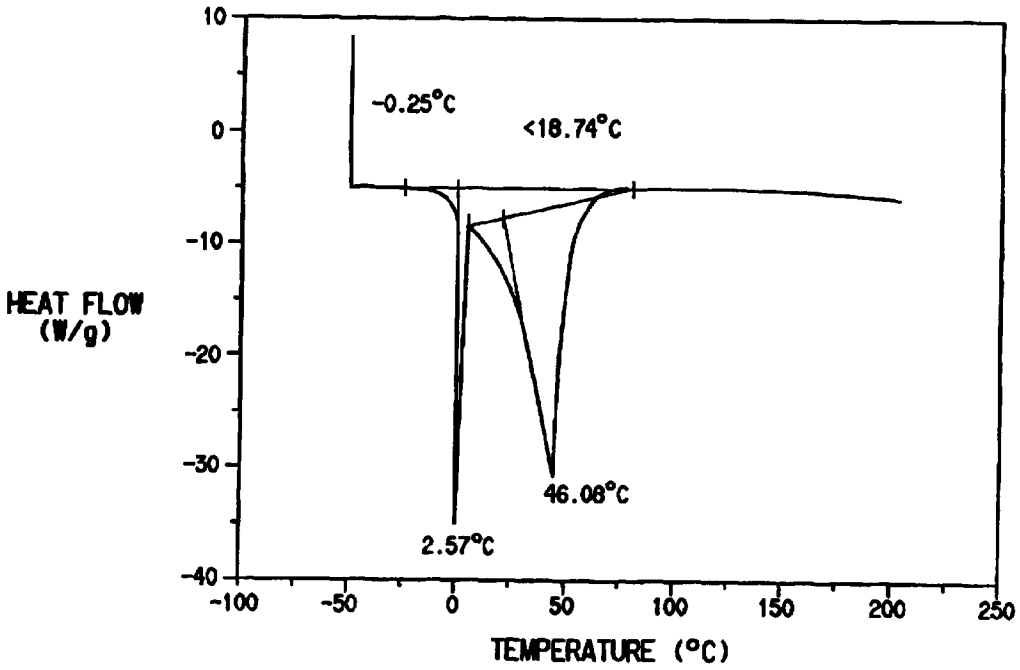
(72) Inventor: UY, William, Cheng; 621 Parkridge Drive, Hockessin, DE 19707 (US).

(74) Agents: BIRCH, Linda, D. et al.; E.I. Du Pont de Nemours and Company, Legal/Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

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(54) Title: PROCESS FOR PRODUCING ZEIN FIBERS



(57) Abstract

The disclosed invention relates to a process wherein zein fibers are prepared by mixing zein and water, preferably between 0 °C and 18 °C, to form a free-flowing, particulate mixture; heating the mixture to a temperature of about 45 °C to about 80 °C while simultaneously extruding the mixture through a spinneret to form zein fibers which are subsequently treated with a formaldehyde stabilizing solution and drawn. This process obviates the need for environmentally unfriendly alkaline solutions and acid coagulating baths.

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TITLE

PROCESS FOR PRODUCING ZEIN FIBERS

FIELD OF THE INVENTION

This invention relates to processes for making fibers from the corn protein, zein. This invention specifically relates to improved, environmentally friendly methods for making zein fibers.

BACKGROUND OF THE INVENTION

Zein is a naturally occurring polymeric protein, obtained as a product of industrial corn processing. Compared to most proteins, zein is characterized by a relative deficiency of hydrophilic groups. In zein, the high proportion of nonpolar and acid amide side chains accounts for the solubility of zein in organic solvents and its classification as a prolamine.

Zein is essentially a globular protein in its natural state. Due to its deficiency of hydrophilic groups, zein does not dissolve easily in aqueous solutions. Hence most known processes to make zein fibers employ an alkaline solution to initially hydrate and dissolve the zein, realigning it as desired (e.g., by spinning) and finally stabilizing the new alignment by inducing cross-linking. Most often the alkaline zein solution is wet spun into acidic coagulating baths. Some processes add formaldehyde to the alkaline zein solutions. After spinning, cross-linking can be induced by treatment in coagulating baths containing formaldehyde, and stabilizing with subsequent treatment with formaldehyde. C. B. Croston et al., describe such a process in "Zein Fibers ... Preparation by Wet Spinning", Industrial and Engineering Chemistry, 37(12) (1945) 1194-1198. Croston et al. call for zein solutions for spinning containing approximately 13 to 16.5% solids, in the pH range of 11.3 to 12.7. In some experiments, denaturing or gelling agents, such as urea

or alcohol were added, or denaturing was effected by applying heat. Formaldehyde was also tested as an additive to the spinning dispersions. The resulting fibers went into a coagulating bath containing sulfuric acid, acetic acid and sometimes zinc sulfate, and were then treated with a mild formaldehyde precuring bath. This precuring bath was found to be necessary prior to the final stretching of the fiber tow which was accomplished in water between two variable speed reels.

The environmental implications of such processes are a major prohibition to commercialization today. The expense of treatment and disposal of the acids, salts and organic compounds required in such processes make production of zein fibers economically impractical.

A few processes have made zein articles from mixtures comprising primarily zein and water. U.S. Patent No. 2,521,738 discloses a process to make artificial bristles from proteins. The process comprises mixing a protein, e.g., casein or zein, with about 80 to 100 percent of its weight of water, and kneading in a mechanical mixer at 80-100°C until a homogeneous plastic gel is obtained. The gel is then converted into fiber bristles by extruding into air and stretching over rolls. The bristles are then treated to subsequent steps of drawing and hardening solutions, which may be a solution of formaldehyde or p-benzo-quinone. This process deliberately effects gelation, since high temperatures and pressures are used, and the bristle fibers formed have tenacities in the range of about 0.8 to about 1.2 grams/denier.

U.S. Patent No. 3,497,369, discloses a composition of zein which is substantially dry and which upon the addition of warm water, at a temperature of about 60°C to about 100°C, forms a pliable plastic composition which may be pulled like taffy, molded, or worked with

as modeling clay. The composition consists of zein and a small portion of plasticizer such as glyceryl monoricinoleate. About 5 parts of plasticizer for every 75 parts of zein is most preferred. The composition can be molded and allowed to harden to form usable articles, such as jewelry.

SUMMARY OF THE INVENTION

This invention provides a process for producing zein fibers, comprising the steps of:

(a) mixing zein with water at a temperature of 0°C to ambient;

(b) heating the mixture to a temperature of about 45°C to about 80°C to form a zein hydrate melt;

(c) forming the zein hydrate melt into zein fibers;

(d) treating said zein fibers with a stabilizing solution; and

(e) optionally, drawing said fibers.

Preferred is a process for producing zein fibers, wherein in step (a) the zein is mixed with water at a temperature of about 0°C to about 18°C to form a free-flowing particulate mixture, and steps (b) and (c) comprise extruding the zein hydrate melt through a spinneret using an extruder having a plurality of zones, the initial zones of said extruder having an ambient temperature and the latter zones having a temperature of about 40°C to about 50°C.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a temperature versus heat flow curve measured on a differential scanning calorimeter (DSC) for a zein/water mixture containing about 65% by weight zein, beginning with the zein mixed with ice at -50°C, to determine the hydration temperature of zein.

Figure 2 shows a temperature versus heat flow curve measured on a differential scanning calorimeter (DSC)

for a zein/water mixture containing about 65% by weight zein, beginning at 0°C to determine the melting point of zein hydrate.

DETAILED DESCRIPTION OF INVENTION

The process of this invention provides a method for making zein fibers which avoids the alkaline solutions of zein and acidic coagulation baths of previous methods. In the process of the present invention, only water need be used to form a spinnable material, and the spun fiber becomes a solid simply through cooling in air. There are no by-products to recover or other waste products. The process of the present invention generally involves the steps of:

(a) mixing zein with water at a temperature of 0°C to ambient;

(b) heating the mixture to a temperature of about 45°C to about 80°C to form a zein hydrate melt;

(c) forming the zein hydrate melt into zein fibers;

(d) treating said fibers with a stabilizing solution; and

(e) optionally, drawing said fibers.

By the term "fibers", as used herein, is meant filaments, fibers, yarns, and threads of any denier or cross-sectional shape and bands or tows consisting of any number of such filamentary articles.

This invention takes advantage of zein hydration formation with rising temperature, by forming the zein mixture into fibers while heating. As shown in Figure 1, a plot of heat flow versus temperature for a zein/water mixture, hydration begins to occur at approximately 18°C. Therefore, when zein is mixed with water above 18°C, generally at ambient temperature (20°C to 32°C) or room temperature (20°C to 25°C), the zein hydrates. Zein can be mixed with water at higher

temperatures, for example between 32°C and 45°C. However, since the zein particles begin to agglomerate and become a sticky, soft, dough-like material at those temperatures (see Table I), it can be difficult to handle or convey the zein hydrate as a feed stock to mechanical means used in fiber formation. Applicant has found that mixing zein and water is easier below ambient temperature.

The present invention demonstrates that preparing a zein/water mixture at a preferred temperature of about 0°C to about 18°C prevents the zein particles from sticking together and allows the mixture to be more easily conveyed or processed. Zein/water mixtures are free-flowing mixtures of solid particles at temperatures between about 0°C and about 18°C, provided the zein and water have not previously formed a hydrate. The use of this lower temperature during preparation facilitates dispersion of the zein in water, which has been problematic in the past where strongly alkaline solutions were required.

One way of forming the zein/water mixture having a temperature between 0°C and about 18°C is by simply spraying ice water onto zein and mixing the two components together. Mixing can be performed by simply kneading by hand or with a stirring rod or by using a conventional mixing apparatus. Placing the mixture in a container and immersing the container in ice water keeps the mixture cold if storage is necessary prior to processing.

By using only water in the mixture with zein, higher zein concentrations can be used. Generally, zein concentrations greater than 50% by weight can be employed in the present invention, and can even range as high as 70%. Preferably, zein hydrate of the present invention will comprise approximately 65% zein by

weight. This is at least a three time higher concentration of zein than the normal 15 to 20% used in previous methods of preparing zein fibers. An advantage to using high concentrations of zein for fiber production is that spinning into air or other gases, becomes possible, in addition to spinning into a liquid. Spinning into a gas can avoid the need for harsh chemical coagulating baths of previous methods. Previous methods using the lower concentrated solutions of zein required spinning into a liquid for the fiber to remain intact.

After mixing, the temperature of the mixture is raised, if necessary, until hydration occurs which is at about 18°C. One way of heating the mixture is by placing the mixture within a container and immersing the container in a hot water bath having a temperature between about 45°C and about 80°C. Another way to heat the mixture is by feeding the zein/water mixture into an extruder having means for heating, such as with electrically controlled zones. As the globular, amorphous zein hydrate structure is formed and then melts, it can be formed into fibers by various mechanical means, typically using melt-spinning methods. Generally, melt spinning is preferable over wet spinning and dry spinning in the industry, in that not only is it a simpler process not requiring coagulation baths or solvent evaporation, but it operates at higher speeds with the resultant fiber displaying more uniform properties throughout.

As the temperature increases, a problem that must be overcome when handling zein hydrate is that of gelation. Zein hydrate will gel with time to give a solid mass that is not readily processable to melt-spinnable fibers thus making it impractical for most commercial fiber purposes. The rate of gelation varies

with the temperature of the zein hydrate. At temperatures of about 80°C or more, zein hydrate will quickly denature and lose its melting and fine fiber drawing properties. For example, 65% by weight zein will denature at 80°C in less than about 15 minutes. (See Example 7). Thus the process of heating and forming fibers must be done in a timely and temperature-conscious manner in order to maintain hydrate stability and avoid gelation. Hydrated zein of the present invention is stable for up to about a week at room temperature or for a few months at about 0°C.

The preferred temperature of the zein hydrate for fiber formation ranges from about 45°C to about 80°C, more preferably from about 55°C to about 63°C. As shown in Figure 2, at a temperature of about 46°C, zein hydrate begins to soften and melt. This zein hydrate melt is highly drawable into fibers and is at a desirable viscosity for spinning. (See Example 1 and Table I.) At temperatures above about 80°C, the zein hydrate melt rapidly loses it stickiness and ductility, making it difficult to draw, and as the temperature increases further, the zein hydrate becomes brittle.

In a preferred embodiment, fiber formation is accomplished by feeding zein and water to an extruder and subsequently to any of the standard spinnerets commonly used in the industry. Spinnerets containing one or a plurality of orifices can be used as the number of orifices in the spinneret has little effect except on the rate of fiber production. As discussed above, it is preferable that zein is mixed with ice water to form a mixture having a temperature between 0°C and 18°C, so the zein particles do not stick together. This free-flowing particulate mixture can than be easily fed into the extruder. Alternatively, the zein and water can be added separately to the extruder by metering both

ingredients into the feed hopper and mixing them together. Separate addition of ingredients to the extruder can provide better mixing and gain time against gelation as compared to premixing. It is preferable that the extruder is of the twin screw type and has a plurality of zones, each zone having its own temperature control. Since at too high temperatures, the tendency of extruders and spinnerets to become fouled and plugged is increased, preferably the initial zones, i.e., those zones nearest the feed hopper, are not heated or are at ambient temperature thus further delaying formation of the hydrate until the mixture reaches the latter zones closer to the spinneret. As the mixture is conveyed along the screws, it is gradually heated to a temperature of about 45°C to about 80°C. The latter zones which are close to the spinneret should be maintained at temperatures which are sufficient to promote hydration and subsequently cause the zein hydrate to melt but not so high that gelation occurs rapidly. Preferably, the temperature of the heated zones is set about 40°C to 50°C. As the mixture is conveyed through the extruder, the mixture attains a higher temperature than that set for the heated zones because of the heat generated through shear. Generally, each zone within the extruder is electrically heated and water jacketed cooled if needed. Additionally, at least the heated zones of the screw extruder should provide sufficient mixing of the zein hydrate in the molten state prior to spinning.

After reaching the appropriate temperature, the zein hydrate melt flows through a transfer line to a spin pump, which meters the melt through a spinneret and extruded as filaments, which are then quenched in the air and may pass through a water bath and onto a winder.

Optionally, plasticizer is added to the water before preparing the zein/water mixture in order to improve flow and, therefore, processability, and to reduce the brittleness of the mixture. Generally about 10 to 15% plasticizer by weight is suitable; about 10% by weight is preferable. Typical plasticizers comprise propylene glycol, and polyethylene glycol having a molecular weight below about 600. Screw type extruders for use with zein/water mixtures with or without plasticizer are commercially available.

The process of forming the zein hydrate and extruding its melt through a spinneret to form fibers effectively orients the zein molecules. Once oriented, the fiber is then stabilized by covalent cross-linking. Cross-linking is accomplished by treating the fibers with a stabilizing solution. By "stabilizing solution" it is meant an aqueous solution comprising cross-linking agents. By "cross-linking agent" is meant any material to which a mixture comprising zein is added that will cause the zein to become cross-linked. By "cross-linked zein" is meant a physical state of zein which will not melt when exposed to hot water. Suitable stabilizing solutions comprise cross-linking agents, such as formaldehyde, as well as optional anti-sticking agents, such as various salts, i.e., sodium sulfate and sodium chloride. The formaldehyde content of the stabilizing solution can be as low as 1% by weight or as high as 5%, preferably, it is 3%. The sodium sulfate can range as high as 20% by weight. The formaldehyde which is employed in the stabilizing solution of the present invention can be added to the stabilizing solution as such or it can be added in the form of materials which yield formaldehyde or react like formaldehyde under the conditions obtained in such stabilizing solutions. For instance, instead of formaldehyde itself, paraformal-

dehyde, trioxymethylene, dimethylthiourea, trimethylol-nitromethane and the like can be used. The fiber is immersed in the stabilizing solution for a time period sufficient to effect stabilization. Generally, this will be at least about 2 hours, though the stabilization time period will also depend on the concentration of crosslinking agent in the stabilizing solution. Temperature can also be a factor. If the fiber has already been collected on a bobbin, the bobbin can be taken off line and rewound passing it through the dilute aqueous formaldehyde solution. Alternatively, formaldehyde can be included in the water used to form the zein hydrate.

Optionally, the fibers can be drawn prior to, during, and/or after treatment in the stabilizing solution to maximize the tensile properties. By drawing, it is meant any stretching of continuous filament yarn or tow to align and further orient the molecules in order to achieve improved tensile properties.

Preferably, there is at least one drawing step after fiber formation, for example by spinning, to achieve the best tensile properties and/or to reduce denier. If the drawing step is accomplished prior to fiber stabilization, drawing should be done in water at ambient temperature, since higher temperatures can melt the fibers. Although the fibers would draw or lengthen at temperatures at or above the melting temperature, they would not achieve molecular orientation. Drawing can also be accomplished after treatment in the stabilizing solution, generally at a draw ratio greater than about 2. Higher temperatures, up to about the boiling point of water, can be used in the water for drawing after the stabilization step since the fibers will not melt at this point. By draw ratio is meant the

amount by which the filaments are stretched following spinning. Generally, the draw ratio is set by controlling the ratio of speeds between the feed roll and the take-up roll of the drawing system. Continuous filaments from a bobbin are wrapped around the feed roll, may pass over a hot pin or other heating device or hot bath, and are wrapped around the take-up roll, which turns faster than the feed roll. The ratio of the take-up roll to the feed roll is the amount of draw or stretching on the filament. Multiple-stage drawing systems are contemplated as a way to further increase tensile properties.

The fibers prepared by the process of this invention are useful in the manufacture of textile materials, for example, in fabrics used for clothing and industrial end usages.

EXAMPLES

EXAMPLE 1

EFFECT OF TEMPERATURE ON ZEIN/WATER MIXTURES

A few grams of zein particles (Freeman Industries, Tuckahoe, NY) were dispersed in a small jar containing excess distilled water. The jar was then placed in a hot water bath and the temperature was raised or lowered according to Table I. The temperature of the jar's content was measured using a thermocouple. The physical characteristics of the zein were observed to change with temperature as follows:

TABLE I
CHANGES IN ZEIN/WATER MIXTURE WITH TEMPERATURE

24°C	Zein particles dispersed easily
32°C	Zein particles began to agglomerate
36°C	Particles completely agglomerated into a dough-like mass
42°C	The dough became softer
50°C	Using tweezers, the zein dough became directly drawable into fine filaments and exhibited sheer opalescence
55-63°C	The zein dough melted to a sticky and highly drawable melt
83°C	Dough lost stickiness and ductility
88°C	Dough became brittle mass
61°C	No change; dough did not regain its melting ability

EXAMPLE 2

PREPARATION OF ZEIN FIBERS

Hydrated zein was prepared as follows. A few grams of zein were placed inside a vial containing excess distilled water at room temperature. The vial was placed in a 60°C water bath. A glass rod was used to stir and knead the hydrated zein dough. Further kneading was done by hand. Zein fibers were hand formed by pulling from the kneaded zein hydrate between two tweezers into a 52°C water bath or heated in 60°C water and quickly drawn in air. The filaments were then treated by immersion in a water bath containing: 3% formaldehyde, 20% sodium sulfate, 4% zinc sulfate, 4% glucose, 2% sulfuric acid and 0.05% cetyl pyridinum bromide. After 20 hours, the filaments were drawn in 88°C to 95°C water. The filaments after treatment with formaldehyde maintained their integrity and were drawn at ratios of 2 to 3x. The drawn filaments were taped to cardboard to prevent shrinkage and then air dried. Fiber tensile (denier per filament (DPF), tenacity, elongation, and modulus properties are shown in Table II.

TABLE II
TENSILE PROPERTIES OF ZEIN FIBER

Fiber Tensile (denier/filament)	Tenacity (grams/denier)	Elongation	Modulus (grams/denier)
159 d	0.31 gpd	47%-83%	14 gpd for 90°C 2x draw
113 d	0.32 gpd	42%	17 gpd for 90°C 3x draw
32 d	0.35 gpd	35%-102%	15-25 gpd for 95°C 3x draw

EXAMPLE 3

EFFECT OF FORMALDHYDE POST-TREATMENT

Hydrated zein was similarly prepared as in Example 2. Zein fibers were formed as in Example 2 in 55-60°C water and immersed in a water bath containing formaldehyde and other components as described in Example 2, for 15 days. Another set of fibers was immersed in a similar bath without the formaldehyde for 15 days. The sample treated without the formaldehyde was weak and fell apart with the slightest tension in cold or hot water. The formaldehyde treated sample was more elastic than those of Example 2, which were treated for lesser time. Maximum draw was 5x in 56°C water. Fiber properties are shown in Table III.

TABLE III
EFFECT OF FORMALDEHYDE TREATMENT ON TENSILE PROPERTIES

	Tenacity (grams/denier)	Elongation	Modulus (grams/denier)
Unstretched	0.31 gpd	19%	18 gpd
2x draw at 56°C	0.76 gpd	28%	29 gpd
3x draw at 56°C	0.62 gpd	9%	20 gpd

EXAMPLE 4

MELTING BEHAVIOR OF ZEIN HYDRATE

This experiment was conducted to determine the melting behavior of zein hydrate. 5.0 grams of zein and

5.0 grams of distilled water were blended and then kneaded at 40-44°C into dough-like consistency. Thermal analysis was carried out using a Differential Scanning Calorimeter (T. A. Instruments, DSC Model 2910, New Castle, DE) and showed a melting range of about of 46°C to about 75°C. See Figure 2.

EXAMPLE 5

DRAWING OF ZEIN FIBERS

Hydrated zein was similarly prepared as in Example 2 except at a temperature of 40°C. Formed fibers, over 12 inches long, were started in 55°C water and completed outside of the water bath. The filaments were first taped down on their ends on plastic films to avoid shrinkage and treated for 24 hours in an aqueous solution containing only 3% formaldehyde while another set of filaments were treated in an aqueous solution containing 3% formaldehyde and 7.6% sodium chloride. The treated filaments were then drawn in 80°C water. The sample treated in the sodium chloride/formaldehyde bath was drawn only about 2-3x and had Tenacity/Elongation/Modulus=0.25 gpd/71%/9 gpd. The sample treated in the formaldehyde bath without sodium chloride was drawn 5x at 56°C to 88°C to study the effect of drawing temperature. Measured fiber properties indicate that drawing temperature in the range studied has little effect on tensile properties (0.44 gpd/28%/20 gpd versus 0.36 gpd/77%/20 gpd for undrawn, for tenacity, elongation, modulus, respectively) other than reducing filament denier. Best sample properties were Tenacity=1.01 gpd, Elongation=25% and Modulus=48 gpd.

EXAMPLE 6

EFFECT OF FORMALDEHYDE TREATMENT TIME

The effect of formaldehyde treatment time is shown by this example. Zein fibers prepared in Example 5 were

treated in an aqueous solvent containing 3% formaldehyde from 1 hour to 18 hours and then drawn in 84°C water. The results are:

TABLE IV
EFFECT OF FORMALDEHYDE TREATMENT TIME

TREATMENT TIME (HRS)	IN 84°C WATER
0 (Control)	Melted
1.0	Intact, but weak
2.0	Intact; drawn 15x
4.0	Intact; drawn 18x
8.0	Intact; drawn 7x
18.0	Intact; drawn 4-5x

EXAMPLE 7

TEMPERATURE/DENATURATION OF ZEIN HYDRATE RELATIONSHIP

This example shows the effect of time and temperature on the denaturation of zein hydrate and its loss of fiber drawing properties. Zein hydrate was prepared as in Example 5, then placed in a constant temperature bath and monitored as to its fiber forming capability. The results are shown in Table V.

TABLE V
TIME TO DENATURE

Temperature °C	Time to Denature
60	< 40 minutes
70	< 17 minutes
80	< 15 minutes

EXAMPLE 8

SPINNING - TWIN SCREW MELT EXTRUDER

A zein hydrate polymer feed, with a composition of 55% zein, 36% water and 9.0% propylene glycol was prepared in batches with each batch prepared as follows: 200 g of zein was weighed into a stainless steel bowl.

While being mixed at the slowest speed setting in a "Sunbeam" mixer, 164 g of cold 20% propylene glycol solution, prepared using ice water, were sprayed into the zein. The components were mixed for another 5 minutes and then stored in a polyethylene container, immersed in ice water. Keeping the mixture cold prevented the particles from sticking together. The zein hydrate polymer feed was manually fed into the feed hopper of a twin screw extruder (Model ZSK-30 available from Werner-Pfleiderer Corp., 663 East Crescent Ave., Ramsey, NJ) maintained at a constant temperature of 45°C except for the first two zones, which were not heated. Spinning was accomplished using a 34 holes/25 mil spinneret with a length to diameter ratio of 4. Bobbins of multi-filament yarn were collected at 16 m/min.

What is claimed is:

1. A process for producing zein fibers, comprising the steps of:

(a) mixing zein with water at a temperature of 0°C to ambient;

(b) heating the mixture to a temperature of about 45°C to about 80°C to form a zein hydrate melt;

(c) forming the zein hydrate melt into zein fibers;

(d) treating said fibers with a stabilizing solution; and

(e) optionally, drawing said fibers.

2. The process of Claim 1 wherein in step (a) the zein is mixed with water at a temperature of about 0° to about 18°C to form a free-flowing particulate mixture.

3. The process of Claim 1 wherein step (c) occurs at a temperature of about 55°C to about 63°C.

4. The process of Claim 2 wherein steps (b) and (c) comprise extruding the zein hydrate melt through a spinneret using an extruder having a plurality of zones, the initial zones of said extruder having an ambient temperature and the latter zones having a temperature of about 40°C to about 50°C.

5. The process of Claim 1 wherein in step (a) the zein comprises about 50% to about 70% by weight of the zein/water mixture.

6. The process of Claim 1 further comprising adding about 10% to about 15% by weight of a plasticizer to the mixture in part (a).

7. The process of Claim 6 wherein the plasticizer is selected from the group consisting of: propylene glycol, and polyethylene glycol having a molecular weight below about 600.

8. The process of Claim 1 wherein the stabilizing solution comprises formaldehyde.

9. The process of Claim 1 wherein step (e) occurs subsequent to step (d) and the fibers are drawn at a draw ratio greater than about 2.

FIG. 1

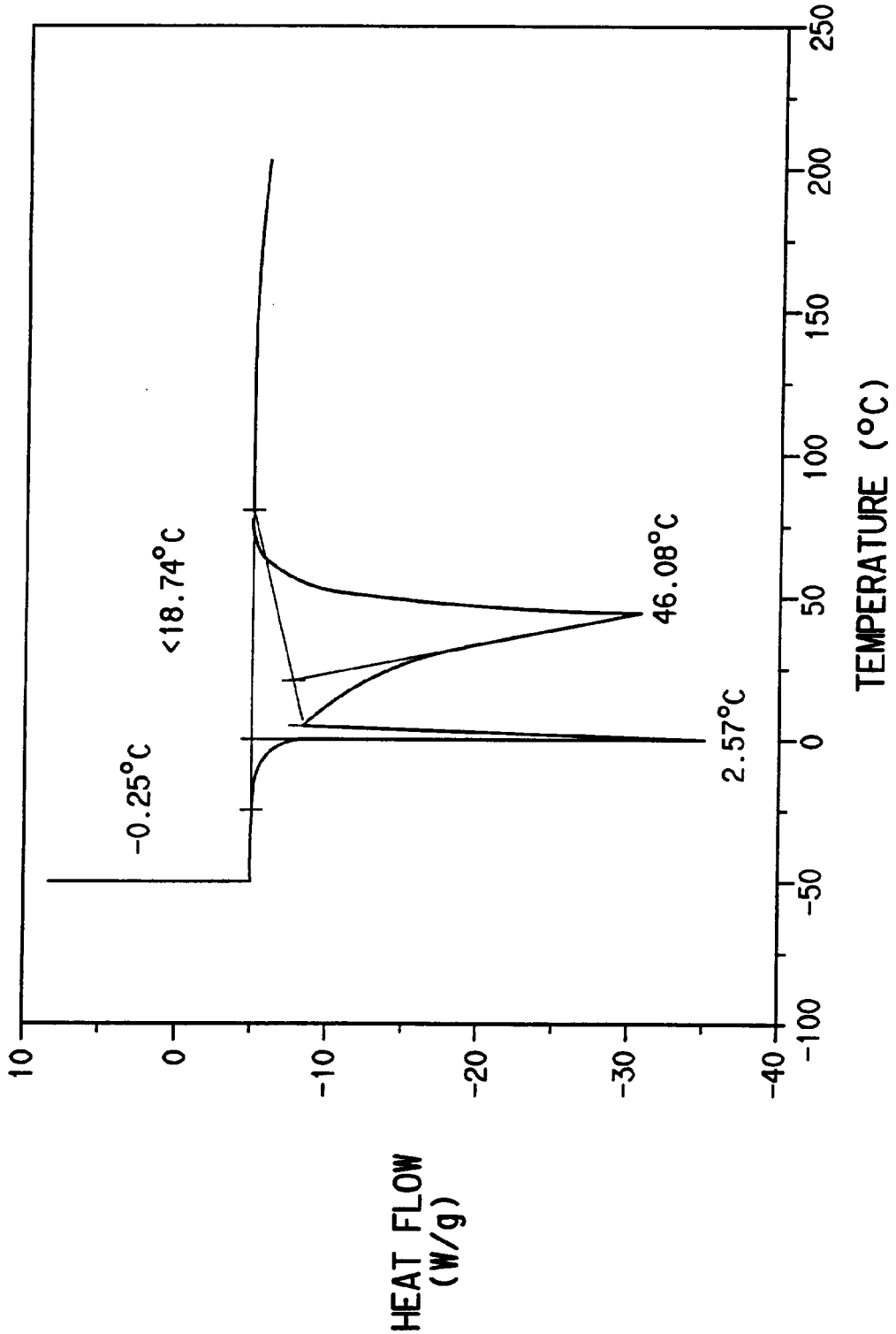
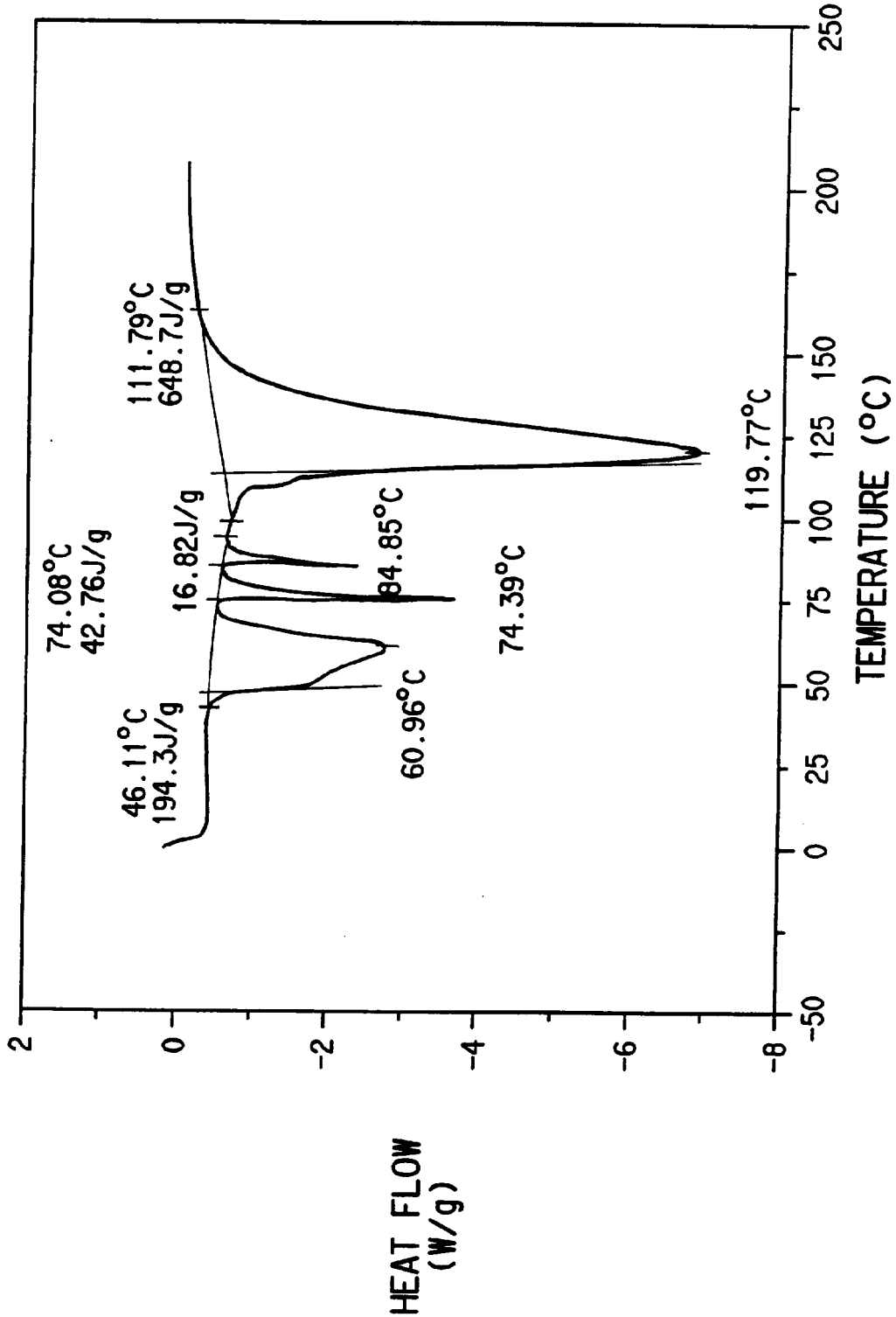

HEAT FLOW (W/g)
10
0
-10
-20
-30
-40
TEMPERATURE (°C)
250
200
150
100
50
0
-50
-100
-0.25°C
<18.74°C
46.08°C
2.57°C

FIG.2


HEAT FLOW (W/g)
2
0
-2
-4
-6
-8
-50
0
25
50
75
100
125
150
200
250
TEMPERATURE (°C)
74.08°C
42.76J/g
46.11°C
194.3J/g
16.82J/g
60.96°C
84.85°C
74.39°C
111.79°C
648.7J/g
119.77°C

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 95/15237

A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 D01F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US,A,2 521 738 (MCMEEKIN THOMAS L ET AL) 12 September 1950 cited in the application see the whole document ---	1-9
A	US,A,3 800 053 (LANGE D) 26 March 1974 ---	
A	US,A,3 870 805 (HAYES LESTER P ET AL) 11 March 1975 ---	
A	US,A,3 953 612 (COPLAN MYRON JULIUS ET AL) 27 April 1976 -----	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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1

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(54) Title: EXPRESSION OF GLOBIN PROTEINS IN PLANTS

(57) Abstract

The present invention relates to genetic-engineering of plants for enhanced oxygen assimilation and utilization. More particularly, this invention relates to producing transgenic plants engineered to express globin proteins such as, for example, hemoglobin, myoglobin, and hemoproteins. The engineered plants of the invention achieve quicker germination, are faster growing or maturing crops, produce higher crop yields, and/or contain higher levels of desired plant metabolites, particularly alkaloids. The invention also relates to mutant *Vitreoscilla* hemoglobin proteins, polynucleotides encoding the same, and host cells containing such polynucleotides.

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EXPRESSION OF GLOBIN PROTEINS IN PLANTS

1. INTRODUCTION

The present invention relates to genetic-engineering of plants for enhanced oxygen assimilation and utilization. More particularly, this invention relates to producing transgenic plants engineered to express oxygen-binding proteins, particularly globins such as, for example, hemoglobin, myoglobin, and hemoproteins. The engineered plants may be used to achieve quicker germination, faster growing or maturing crops, higher crop yields and/or plants with higher levels of desired plant metabolites, particularly alkaloids.

2. BACKGROUND OF THE INVENTION

Genetic engineering of plants through transgenic approaches has rapidly become a diverse and promising field. Transformation techniques are applied both to study plant development and physiology, and in attempts to generate plants with advantageous agricultural properties.

For example, much effort has focused on improving crop production by transforming plants with herbicide or pesticide resistance genes. When grown under field conditions, the resulting transgenic plants can tolerate and survive applications of herbicides or pesticides which would destroy non-transformed plants. Alternatively, plants themselves have been genetically engineered to express products which are shown to act as natural insecticides such as *Bacillus thuringensis* toxin proteins (*see* U.S. Patent No. 5,380,831, issued Jan. 10, 1995).

Genetic engineering has also been attempted to generate plants with altered compositions of primary metabolites such as starch, sugar, and oils. For example, a bacterial levan sucrase gene was transformed into plants to increase levels of fructans in the resulting transformant. In another approach, the activity of certain enzymes involved in the metabolic synthesis of oils has been decreased using antisense technology in order to design vegetable oils exhibiting the desired properties. (*Reviewed in* Knauf, 1995, Curr. Opin. Biotech. 6:165-70).

In addition to attempts to alter primary metabolic compositions of plants (*i.e.* carbohydrates, oils, amino acids and proteins), it would also be desirable to alter secondary metabolite compositions. Secondary metabolites are those specialized plant products required by cells in small amounts (*e.g.* hormones), or

are highly specialized biomolecules (*e.g.* nucleotides, pigments, toxins, antibiotics, and alkaloids). Generally, secondary metabolites can be defined as compounds that have no recognized role in the maintenance of fundamental life processes of the cell, but which may perform other advantageous functions for the organism as a whole. (*See* for example, Bell, 1981, "The Physiological Role(s) of Secondary (Natural) Products" in The Biochemistry of Plants, v. 7, p. 1, Academic Press, Inc.)

The production of useful secondary metabolites from plants and plant cell cultures is an important aspect of plant technology. About 25% of the prescription drugs used in the industrialized world contain ingredients extracted from higher plants (Tyler, V.E., 1988, Planta Med. 54:95-100). The most prominent group of plant substances used in medicine are the alkaloids (Nickell, L.G., 1980, "Products," Plant tissue culture as a source of biochemicals, Staba, E.J., Ed., Boca Raton, CRC Press Inc., 235-269). Alkaloids are secondary metabolites which protect plants against phytophagocytosis by higher organisms or invasion by pathogens (Robins, R.J. *et al.*, 1991, Planta Med. 57:27-35). Plant alkaloids are generally derived from simple amino acids that interact with acetate and terpenoid units and undergo aromatic hydroxylations (Robins, *supra*). Examples of plant alkaloids are nicotine, scopolamine, hyoscyamine, ajmalicine, serpentine, piperine, leucenol, mimosine, ricinine, pelletierine, cocaine, hygrine, lupinine and anagyrine.

Several examples of introducing a new metabolic pathway or redirecting previously existing ones to increase production of secondary metabolites have been described (Bailey, *supra*; Berlin, J. *et al.*, 1994, Stud. Plant Sci. 4:57-81; Lilius, G., Holmberg, N. and Bülow, L., 1996, Bio/Technology 14:177-180). However, apparently all of these genetic engineering approaches entail either the up-regulation of a limiting enzyme in the pathway (for example through transformation and expression of the cloned gene), or the down-regulation of competing enzymatic pathways (for example through anti-sense technology). (*See* for review, Robins et al., 1991, Planta. Med. 57:27-35.)

Plant cell suspension cultures have often been used for industrial production of plant secondary metabolites. During *in vitro* culture, a critical parameter influencing secondary metabolite formation, especially in scale-up cultures, is the

level of dissolved oxygen. For instance, it has been demonstrated that a high dissolved oxygen concentration promotes the production of ajmalicine in *Catharantus roseus* batch cultures (Schaltmann, J.E. *et al.*, 1995, Biotech. Bioeng. 45:435-439). Similarly, increased alkaloid levels with increasing aeration have been observed in *Berberis wilsonae* (Breuling, M. *et al.*, 1985, Plant Cell Rep. 4:220-223). From these results it has been suggested that oxygen is a limiting substrate in plant tissue culture secondary metabolism, as well as for primary metabolism, especially for industrial scale-up processes. However, this need for increased oxygen has always been thought to be a need for extracellular oxygen during fermentation batch processes.

Aerobic metabolism has been successfully enhanced in fermentation microorganisms and cultured mammalian cells by engineering the host to express the *Vitreoscilla* hemoglobin gene (VHb) (Khosla, C., Bailey, J.E., 1988, Nature 331:633-635; Khosla, C., Bailey, J.E., 1988, Mol. Gen. Genet. 214:158-161; DeModena, *supra*; Chen, W. *et al.*, 1994, Biotechnol. Prog. 10:308-313; Pendse, G.J. and Bailey, J.E., 1994, Biotechnol. Bioeng. 44:1367-1370). This metabolic engineering strategy has been shown effective, for example, in increasing total cell protein synthesis by oxygen-limited *Escherichia coli* (Khosla, C. *et al.*, 1990, Bio/Technology 8:849-853 and U.S. Patent No. 5,049,493, issued Sept. 17, 1991), improving lysine yield and titer in cultivations of *Corynebacterium glutamicum* (Sander, F. *et al.*, 1994, Proc. 6th Eur. Congress Biotechnol., Alberghina, L., Frontali, L. and Sensi, P. (eds.) Elsevier Science B.V., Amsterdam 607-610), and in increasing actinorhodin and cephalosporin C production in *Streptomyces coelicolor* and *Acremonium chrysogenum*, respectively (Magnolo, S.K. *et al.*, 1991, Bio/Technology 9:473-476; DeModena, J.A. *et al.*, 1993, Bio/Technology 11:926-929).

Hemoglobin and globin-like proteins exist ubiquitously in mammals, and are less frequently found in plants. The few plant hemoglobin-like molecules described, which include lupin and soybean leghemoglobin, are largely thought to be associated with nitrogen-fixation activities of these plants, although some researchers claim that hemoglobin-like proteins occur in the roots of all plants. However, in no case have these proteins been overexpressed in plants. Although several major plant metabolic pathways are known to be oxygen-dependent,

including chlorophyll and heme biosynthesis, fatty acid desaturation and cysteine, glycine and serine biosynthesis, oxygen has not been thought to be a limiting factor in metabolism in intact plants. Rather, the observed effects of dissolved oxygen on metabolism in plant tissue cultures has been attributed to mass transport problems during large-scale, or even batch culture, fermentations (*see* Schlatmann et al., 1991, *supra*.). Thus, until the present invention, the advantages of altering useable levels of intracellular oxygen in intact plants have not been recognized.

3. SUMMARY OF THE INVENTION

The present invention is directed to the generation of plants with improved agronomic or medicinal characteristics by metabolically engineering an increased intracellular oxygen level or increased utilization of oxygen. Such engineered plants display enhanced production of valuable secondary metabolites, in particular those metabolites which are synthesized by oxygen-dependent pathways, as well as other unexpected and valuable properties such as more rapid germination, higher levels of chlorophyll, increased overall growth metabolism, and increased rate of accumulation of biomass.

The invention encompasses both genetically engineered plants and methods of producing the same. Therefore, in one of its embodiments, the invention encompasses a method of metabolically engineering plants by increasing intracellular levels of oxygen or intracellular utilization of oxygen. The invention includes a method of producing a plant with an improved agronomic or medicinal characteristic by transforming the plant with polynucleotides encoding an oxygen-binding protein. Particularly preferred oxygen-binding proteins are globins, which reversibly bind oxygen through a heme group. In an exemplary embodiment, the oxygen-binding protein is *Vitreoscilla* hemoglobin. Preferably, the polynucleotides encoding the oxygen-binding protein are operably linked to a strong, constitutively expressed plant promoter, for example, the CaMV 35S promoter. Another aspect of the invention is a plant or plant cell which has been genetically engineered by the methods of the invention. The invention encompasses plants and plant cells which are both dicotyledons and monocotyledons.

Still another aspect of the invention is mutant *Vitreoscilla* hemoglobin proteins, polynucleotides encoding the same, and host cells containing such mutant

proteins and polynucleotides. The host cells can be bacteria, plant, animal, fungi or algae. The invention also encompasses a method of generating polynucleotides encoding mutant *Vitreoscilla* hemoglobin proteins. The method entails amplifying a polynucleotide that encodes *Vitreoscilla* hemoglobin protein using a low fidelity polymerase chain reaction, transforming the amplified polynucleotides into bacteria, and selecting bacteria that contain a polynucleotide sequence encoding the mutant *Vitreoscilla* hemoglobin protein. In a preferred aspect of this method, bacterial colonies containing mutant *Vitreoscilla* hemoglobin proteins are selected by virtue of their color phenotype. Polynucleotides encoding mutant *Vitreoscilla* hemoglobin proteins that are isolated by this method, as well as the mutant proteins themselves, and host cells containing such polynucleotides, are also within the scope of the present invention.

4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1: FIG. 1A. The last steps in the *Nicotiana tabaccum* biosynthesis of the secondary metabolites nicotine and anabasine. The reaction catalyzed by nicotine synthase, the oxygen-dependent enzyme catalyzing the conversion of nicotinic acid and N-methyl-Δ'-pyrrolinium salt into nicotine, is labelled in bold. FIG. 1B. Oxygen-dependent steps in chlorophyll and heme biosynthesis in plants. The last oxidation of chlorophyll a to chlorophyll b has only been observed *in vitro* (Scheer, H. (ed.), 1991, Chlorophylls CRC Press, Boca Raton, FL, USA 421-425).

Figure 2. Construction of the Ti-expression vector containing the *Vitreoscilla* VHb gene using PCR. The gene was placed under control of the CaMV 35S promoter and transcription termination was achieved by the NOS region. The approximate positions of the PCR primers are indicated by solid arrows. Right and left border of the T-DNA are indicated by RB and LB, respectively. NOS-prom, NPT II and NOS are abbreviations for the nopaline synthase promoter, the neomycine phosphotransferase II gene, and the nopaline synthase 3' transcription termination region, respectively.

Figure 3. Dried weight measurements at different time intervals are shown as mean values. Bars correspond to the standard error. The data from the first two time points (15 and 20 days) correspond to 12 plants but weighed in triplets,

and the data from the last two time points are from 8 plants individually weighed. This experiment was duplicated and the data points were subjected to statistical analysis using one-way analysis of variance (ANOVA).

Figure 4. Nicotine and anabasine production in the transgenic lines, He3 and He4, and a wild-type line. The nicotine concentrations are given μg/g fresh weight and the anabasine concentrations as μg/g freeze-dried plant. The nicotine and anabasine concentrations were measured in eight plants and six plants, respectively. All measurements were performed in duplicate, with the standard deviations being less than 1% and 25%, respectively.

Figure 5. The chlorophyll contents in the two transgenic tobacco lines and a wild-type control measured as μg chlorophyll/ml x mg fresh weight. The bars represent the mean values of 18 plants. The standard errors of the mean were less than 10%, 8% and 5% for wild-type tobacco, Hc3 and He4, respectively.

Figure 6. Schematic map of the pUC derivative plasmid pJFK2 used for transformation of rice.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to metabolic genetic engineering by effectively increasing the intracellular level of available oxygen in plants. The invention is based, in part, on the discovery that transforming plants so as to express oxygen-binding proteins, and in particular heme-containing globins, improves the properties of the transformed plants in unexpected ways. The resulting plants produce higher levels of secondary metabolites whose production is oxygen sensitive. Surprisingly, however, these genetically engineered plants also exhibit better growth characteristics, more rapid germination, and improved vegetative yield. Without intending to be limited to a particular mechanism, it is believed that these surprising results are due to either an increased level of intracellular oxygen, or an increased efficiency in intracellular oxygen utilization.

Accordingly, the targets for engineering are genes encoding proteins involved in binding oxygen, particularly those which bind oxygen reversibly such as the globins. The target genes include, but are not limited to, those encoding *Vitreoscilla* hemoglobin (VHb), horse heart myoglobin, E. coli hemoprotein, B.

subtilis hemoprotein, yeast flavohemoglobin, soybean leghemoglobin, lupin leghemoglobin, and sperm whale myoglobin. In fact, an engineered increased expression of any endogenous plant hemoglobin is also within the scope of the invention.

Particularly suitable for use in the present invention are those oxygen-binding proteins which have relatively high k_{off} rates such as VHb (k_{off} 5600 s^{-1}; Orii and Webster, 1986, J. Biol. Chem. 261:3544-3547) or relatively low oxygen affinity such as horse heart myoglobin (K_D 0.79 μM; Wittenberg et al., 1985, in Nitrogen fixation research progress, H.J. Evand et al. Eds. Martinus Nijhoff Publishers , Dordrecht, p. 354). Therefore, particularly preferred oxygen binding proteins may be those proteins with a k_{off} rate for oxygen of greater than 10 s^{-1}, or a K_D less than 0.5 μM, although it will be understood that oxygen-binding proteins with rate constants outside of these parameters will also be useful. Other examples of preferred oxygen-binding proteins are globins such as hemoglobin, myoglobin, and leghemoglobins. The properties of many oxygen-binding proteins, including globins, are disclosed in the literature. Additionally, techniques for determining the oxygen-binding properties of a protein such as a globin are well known to one of skill in the art and may be performed without undue experimentation.

A particularly advantageous oxygen-binding protein for use in the instant invention, as described herein by way of working example, is *Vitreoscilla* hemoglobin ("VHb"). The complete sequence of the VHb gene is described in U.S. Patent No. 5,049,493, *supra*. Mutants of VHb which bind oxygen are also within the scope of the present invention. As described more fully below by way of a working example, genes encoding functional mutants of VHb are generated through PCR mutagenesis, and then tested in a simple phenotypic screen for their ability to bind oxygen. Using these techniques, several VHb mutants have been obtained for use in the present invention.

Also encompassed within the scope of the invention are any nucleotide sequence which hybridizes to the complement of the nucleotide sequence of the VHb gene under highly stringent conditions, *e.g.*, hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing

Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a functionally equivalent gene product; and any nucleotide sequence that hybridizes to the complement of the nucleotide sequence of the VHb gene under less stringent conditions, such as moderately stringent conditions, *e.g.*, washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989, *supra*), yet which still encodes a functionally equivalent Vhb gene product. Functional equivalents of VHb include naturally occurring hemoglobin genes present in other species, and mutant VHb whether naturally occurring or engineered which retain at least some of the functional activity of VHb (*i.e.*, reversible oxygen binding). Species from which genes encoding functional equivalents of VHb include but are not limited to bacterial species, and in particular those bacterial species found in soil, dung, and in other oxygen-accessible, but poorly aerated, environments.

Furthermore, using the teachings of the present invention one of skill in the art can also isolate genes encoding other bacterial proteins which reversibly bind oxygen through a heme group without relying upon sequence homology with known proteins. For example, one may isolate and culture a bacterial organism, prepare genomic DNA from the cultured bacteria, and then construct an expression library with the DNA in *E. coli* using well known techniques. The expression library is plated to obtain isolated colonies. Those *E. coli* colonies carrying plasmids which expresses an oxygen-binding heme protein may be identified phenotypically by their red color. The expression library DNA clones encoding oxygen-binding proteins may be further characterized and subcloned into plant expression vectors. The expression of such oxygen-binding heme proteins in plants is accordingly encompassed by the invention.

The invention also encompasses degenerate variants of nucleotide sequences which encode the amino acid sequence of the VHb protein, VHb mutants, and functional equivalents of VHb encoded by nucleotide sequences which hybridize to the complement of the nucleotide sequence of the VHb gene. For example, the nucleotide sequence may be altered so as to optimize amino acid codon usage for expression in the chosen host cell.

The levels of oxygen-binding proteins can be altered or their expression can be enhanced or otherwise modified (*e.g.*, ectopic expression or tissue specific expression) to engineer a plant with desirable properties. The engineering is

accomplished by transforming plants with nucleic acid constructs described herein. The transformed plants or their progenies are screened for plants that express the desired protein or exhibit the desired altered expression of the oxygen-binding protein, altered expression of the corresponding mRNA, altered secondary metabolite levels, increased growth rate, enhanced vegetative yield and/or increased germination rate.

Engineered plants exhibiting the desired physiological and/or agronomic changes can be used in plant breeding or directly in agricultural production. These plants having one altered protein also may be crossed with other altered plants engineered with alterations in other pathways (*e.g.*, cross an oxygen-binding expressing plant to a plant which has been otherwise genetically engineered) to produce lines with even further enhanced physiological and/or agronomic properties compared to the parents.

The invention is illustrated by working examples of plants engineered for expression of *Vitreoscilla* hemoglobin (VHb). In all instances, engineered plants that exhibit expression also show better growth characteristics, increased rate of seed germination, improved vegetative yield and/or enhanced levels of secondary metabolites over control, wild-type plants.

The invention is based, in part, on the surprising discovery that transformation of plants with oxygen-binding proteins provides several advantages. Plants were transformed with expression plasmids encoding an oxygen-binding protein in an attempt to develop plants with increased drought tolerance. As a model system, *Nicotiana tabaccum* (tobacco) was first chosen to be transformed with expression constructs encoding the oxygen-binding protein *Vitreoscilla* hemoglobin (VHb). However, since there is an oxygen-dependent step in the nicotine biosynthesis pathway, the production of nicotine was examined.

The VHb gene was introduced into *Nicotiana tabaccum* (tobacco) via *Agrobacterium* mediated gene transfer. Transcription and translation of the VHb gene and message were demonstrated by reverse transcriptase PCR and Western immunoblot analysis, respectively. Transgenic tobacco plants expressing VHb exhibited on average 34% greater nicotine content than wild-type controls. They also contained an altered distribution of nicotine relative to the less desirable alkaloid anabasine. Additional effects of VHb expression on the physiology of the

transgenic tobacco plants were also noted; these plants contained on average 10-20% more chlorophyll than controls. Moreover, the plants germinated more quickly and exhibited a more rapid rate of dry weight increase. Thus, engineering of plants to express additional oxygen-binding proteins not only produced increased alkaloid levels and altered distributions of alkaloids, but also enhanced plant growth.

In yet another illustration of the advantages of the present invention, plants from the genus *Datura* were transformed with the same expression construct used to transform the tobacco plants. *Datura* is the major commercial source for production of the secondary metabolites scopolamine and hyoscyamine, which are used as sedatives and as starting material for semisynthetic derivatives of these metabolites. Surprisingly, scopolamine and hyoscyamine levels in the transgenic plants were increased as much as 5 to 6 fold over levels found in non-transformed plants.

In still another illustration of the invention, plants from the genus *Oryzae* were transformed by the ballistic method with a polynucleotide construct that directs the expression of the VHb hemoglobin gene. Thus, the present invention is generally applicable for altering the oxygen utilization in all plant types in order to obtain plant varieties with advantageous medicinal and agronomic qualities. For example, plants which germinate more quickly and accumulate biomass more rapidly will find particular application in agricultural environments where arable land and/or the length of growing season are limiting factors to production. Even a small increase in biomass production and/or rate of growth can prove significant. Additionally, the increased levels of economically important secondary metabolites may enable production of metabolites from previously economically unfeasible plant sources.

Further, the invention encompasses a method of generating polynucleotides encoding mutant *Vitreoscilla* hemoglobin proteins. The method entails amplifying a polynucleotide that encodes *Vitreoscilla* hemoglobin protein using a low fidelity polymerase chain reaction, transforming the amplified polynucleotides into bacteria, and selecting bacteria that contain a polynucleotide sequence encoding the mutant *Vitreoscilla* hemoglobin protein. In a preferred aspect of this method, bacterial colonies containing mutant *Vitreoscilla* hemoglobin proteins are selected

by virtue of their color phenotype; a red or pink color indicates that the bacteria express an oxygen-binding heme protein. Polynucleotides encoding mutant *Vitreoscilla* hemoglobin proteins that are isolated by this method, as well as the mutant proteins themselves, and host cells containing such polynucleotides, are within the scope of the present invention. The host cells can be bacteria, plant, animal, fungi or algae. Such mutant *Vitreoscilla* hemoglobin proteins are useful in any number of applications for which wild-type *Vitreoscilla* hemoglobin proteins are advantageous, including but not limited to increasing cell growth and increasing cell production.

5.1. PRODUCTION OF TRANSGENIC PLANTS

According to the present invention, a desirable plant or plant cell may be obtained by transforming a plant cell with the nucleic acid encoding an oxygen-binding protein, particularly a globin, and in one embodiment VHb, as described herein. In some instances, it may be desirable to engineer a plant with several different gene constructs. Such engineering may be accomplished by transforming a plant or plant cell with all of the desired gene constructs simultaneously. Alternatively, the engineering may be carried out sequentially. That is, transforming with one gene construct, obtaining the desired transformant after selection and screening, transforming the transformant with a second gene construct, and so on.

In an embodiment of the present invention, *Agrobacterium* is employed to introduce the gene constructs into plants. Such transformations preferably use binary *Agrobacterium* T-DNA vectors (Bevan, 1984, Nuc. Acid Res. 12:8711-8721), and the co-cultivation procedure (Horsch et al., 1985, Science 227:1229-1231). Generally, the *Agrobacterium* transformation system is used to engineer dicotyledonous plants (Bevan et al., 1982, Ann. Rev. Genet. 16:357-384; Rogers et al., 1986, Methods Enzymol. 118:627-641) The *Agrobacterium* transformation system may also be used to transform, as well as transfer, DNA to monocotyledonous plants and plant cells (*see* Hernalsteen et al., 1984, EMBO J. 3:3039-3041; Hooykass-Van Slogteren et al., 1984, Nature 311:763-764; Grimsley et al., 1987, Nature 325:1677-179; Boulton et al., 1989, Plant Mol. Biol. 12:31-40; Gould et al., 1991, Plant Physiol. 95:426-434).

In other embodiments, various alternative methods for introducing recombinant nucleic acid constructs into plants and plant cells may also be utilized. These other methods are particularly useful where the target is a monocotyledonous plant. Alternative gene transfer and transformation methods include, but are not limited to, protoplast transformation through calcium-, polyethylene glycol (PEG)- or electroporation-mediated uptake of naked DNA (*see* Paszkowski et al., 1984, EMBO J 3:2717-2722, Potrykus et al., 1985, Mol. Gen. Genet. 199:169-177; Fromm et al., 1985, Proc. Natl. Acad. Sci. USA 82:5824-5828; Shimamoto, 1989, Nature 338:274-276) and electroporation of plant tissues (D'Halluin et al., 1992, Plant Cell 4:1495-1505). Additional methods for plant cell transformation include microinjection, silicon carbide mediated DNA uptake (Kaeppler et al., 1990, Plant Cell Reporter 9:415-418), and microprojectile bombardment (*see* Klein et al., 1988, Proc. Natl. Acad. Sci. USA 85:4305-4309; Gordon-Kamm et al., 1990, Plant Cell 2:603-618).

According to the present invention, a wide variety of plants may be engineered for the desired physiological and agronomic characteristics described herein using nucleic acid constructs encoding oxygen-binding proteins and the various transformation methods mentioned above. Target plants for engineering include, but are not limited to, those of tobacco, *Datura*, maize, wheat, rice, soybean, tomato, carrots, peanut, potato, sugar beets, sunflower, yam, *Arabidopsis*, rape seed, petunia and other ornamental flowers, turfgrass, barley, alfalfa, potato, cassava, sweet potato, lettuce, sugar cane, fruit trees (*e.g.* cherry, apple, peach, pear, plum, orange, lemon), nut trees (*e.g.* walnut, almond), banana, peanut, strawberry, cauliflower, artichoke, cucumber, melon, grape, pepper, cabbage, endive, leek, lettuce, spinach, arrowroot, radish, bean and pea.

According to the present invention, desired plants may be obtained by engineering the gene constructs described herein into a variety of plant cell types, including but not limited to, protoplasts, tissue culture cells, tissue and organ explants, pollen, embryos as well as whole plants. In an embodiment of the present invention, the engineered plant material is selected or screened for transformants (*i.e.*, those that have incorporated or integrated the introduced gene construct(s)) following the approaches and methods described below. An isolated transformant may then be regenerated into a plant. Alternatively, the engineered